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Casein Kinase 2 Controls Synapse Organization and Stability

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CONTENT

SUMMARY

Synapse remodeling is a local and dynamic process, requiring constant modifications of the neuronal network. Post-translational modifications, such as reversible phosphorylation by kinases and phosphatases, play a crucial role in synapse development and stabilization (Caroni et al., 2012; Wu et al., 2010). To better understand the complex regulation of synaptic stabilization, I performed an unbiased genome-wide RNAi screen consisting of 312 kinases and 127 phosphatases or their subunits. Seven kinases and four phosphatases not previously implicated in synapse stability were identified. They control synapse stability through modulation of phospholipid signaling, metabolic signaling, or the cytoskeleton. The analysis of a major regulatory molecule identified in the screen Casein Kinase 2 (CK2) provides novel insights into the mechanisms controlling the regulation of synapse stability.

CK2 is largely conserved and constitutively active, consisting of two catalytic CK2α and two regulatory CK2β subunits. I demonstrated that both kinase-active CK2α and regulatory CK2β are indispensable for synapse stability pre- but not postsynaptically at the Drosophila neuromuscular junction (NMJ). Moreover, CK2α-CK2β interaction is necessary for the maintenance of stable synapses *in vivo* demonstrating that CK2 functions as an α2/β2 holoenzyme. I identified essential residues in CK2α that mediate this interaction. In addition, I showed that presynaptic CK2α activity regulates synaptic domain organization and localization of essential synaptic proteins such as the cell adhesion molecule Neuroglian (Nrg) and the adaptor molecule Ankyrin2 (Ank2). Ank2 provides a regulatable link between synaptic cell adhesion molecules and the actin and microtubule cytoskeleton. I demonstrated that CK2 phosphorylates Ank2 *in vitro* and controls synapse stability at least partially via Ank2, but not via its interaction with Protein Phosphatase 2A (PP2A). Therefore, Ank2L represents an essential substrate of CK2 to regulate synaptic morphology and stabilization. CK2 may thus be an important protein kinase whose function is to control synapse stability through constitutive phosphorylation of structural synaptic components. This provides a compelling mechanism to regulate the lifetime of synaptic connections and to enable local structural synaptic plasticity without disruption of neuronal circuit architecture.

INTRODUCTION

Role of Synapses in the Nervous System

Synapses are specialized macromolecular junctions between cells that mediate transmission of information. The presynaptic axon terminals contain vesicles filled with neurotransmitters and the machinery ensuring their release. The postsynaptic specializations in other neurons or non-neuronal cells include transmembrane neurotransmitter receptors, scaffold proteins, and signaling machinery. Synapses are fundamental signaling components of the nervous system that regulate intercellular communication in the nervous system, participate in the computation of neuronal networks, and store information through activity-dependent structural modifications.

Synapse development is a multi-step process including synapse formation, maturation, and stabilization (Li and Sheng, 2003; Lin and Koleske, 2010). Synapse formation starts during the final stages of embryogenesis and involves pairing of the pre- and postsynaptic partners. Numerous cellular and genetic mechanisms strictly control this process, which requires the precise execution of multiple developmental steps. These steps include cell fate specification, cell migration, axon guidance, synaptic target selection, and synaptogenesis (Juttner and Rathjen, 2005; Salie et al., 2005; Waites et al., 2005). Synapse maturation is distinguished by an increase in its morphological size and the strength of transmission. Importantly, synaptic activity determines whether newly formed synapses are stabilized or eliminated. The apparent stability of synaptic contacts could be viewed as a balance between growth and retraction. Even in the mature central nervous system, changes in synapse structure and function continues to be a highly dynamic process contributing significantly to learning and memory as well as other adaptive abilities of the brain (Caroni et al., 2012; Xu et al., 2009; Yang et al., 2009).

Although the molecular mechanisms of synapse formation have been relatively well investigated, much less is known about mechanisms that stabilize synaptic connections. Equally little is known about mechanisms that disassemble previously functional synapses (Goda and Davis, 2003; Li and Sheng, 2003; Lin and Koleske, 2010). Interestingly, it is known that activity-mediated spine plasticity can be regulated locally. It has been demonstrated that new dendritic

spines tend to be established in clusters, as they prefer to form in close proximity to already existing activated spines (De Roo et al., 2008; Fu et al., 2012; Harvey et al., 2008). In addition, live imaging studies have shown that single motorneurons can disassemble synapses at some muscles while at the same time increase the size of synaptic connections at other muscle targets (Kasthuri and Lichtman, 2003; Keller-Peck et al., 2001). The fact that synapse growth can occur locally and often coincidentally with synapse disassembly suggests that local post-translational modifications might be needed to execute the switch. Therefore, our knowledge of how synapse formation and elimination are regulated is critical to our understanding of the nervous system and how it goes awry in neurological disorders.

Molecular Mechanisms of Synapse Stabilization in Mammals. Regulation by Kinases and Phosphatases

The molecular mechanisms responsible for synapse stabilization and disassembly involve a variety of factors. First, various signaling pathways and actin-regulatory proteins controlling the actin cytoskeleton are essential for dendritic spine stabilization. Long-term potentiation (LTP) and spine size modulation are impaired if actin polymerization process is perturbed (Bramham, 2008; Cingolani and Goda, 2008; Honkura et al., 2008). Second, synapse stability can be modulated by changes in the organization of postsynaptic density (PSD) that support trans-synaptic adhesion and contact. For example, PSD-95 is required for activity-driven synapse stabilization (Ehrlich et al., 2007). Likewise, expression of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors, which mediate fast excitatory synaptic transmission in the brain, improves synaptic strength and stability (Ripley et al., 2011). It has also been demonstrated that several cell adhesion molecules, such as Neuroligin 1 and N-cadherin, are crucial for regulating presynaptic maturation and plasticity-induced long-term synapse stabilization (Mendez et al., 2010; Ripley et al., 2011; Wittenmayer et al., 2009). Third, synapse stabilization can be regulated through the local control of *de novo* RNA and protein synthesis. This mechanism includes such signaling cascades as the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-Akt, and phospholipase Cγ (PLC-Cγ)-Ca^{2+} pathways downstream of receptor tyrosine kinase B (TrkB) activation, the mammalian target of rapamycin (mTOR) signaling complex, and the translation of the appropriate mRNAs (Caroni et al., 2012; Minichiello, 2009). Fourth, post-translational modifications, such as phosphorylation

and de-phosphorylation, play a crucial role in all of the steps described above. This indicates the importance of learning about the post-translational modification mechanisms, as synapse remodeling is a local and dynamic process, requiring constant changes of the neuronal network.

One kinase that is directly involved in LTP maintenance and learning is calcium/calmodulin-dependent protein kinase II (CaMKII) (Lisman et al., 2012). CaMKII is a major synaptic protein which plays an essential structural role in an activity-mediated enlarging and strengthening of the synapses (Lisman et al., 2012; Yamagata et al., 2009). Indeed, many mutant mice in which CaMKII activity is increased show enhanced learning, and the opposite is also true (Lee and Silva, 2009; Lledo et al., 1995; Pi et al., 2010). One constitutively active isoform of the Protein Kinase C (PKC) – PKMζ - was also thought to be essential for maintaining long-term memories (Sacktor, 2011). However, the PKCζ molecule memory theory was recently debunked, as PKCζ null mice, surprisingly, showed normal learning and memory (Lee et al., 2013; Volk et al., 2013). Regardless, PKC is directly involved in synapse stabilization *in vivo* (Bednarek and Caroni, 2011). It performs its role via phosphorylating the cytoskeleton-stabilizing protein β-adducin, and this process is required for the stabilization of synapses induced by environmental enrichment (Bednarek and Caroni, 2011). Interestingly, we originally demonstrated that the Drosophila homologue of β-adducin is required for synapse stability at the Drosophila neuromuscular junction (NMJ) and is regulated in a phosphorylation-dependent manner (Pielage et al., 2011).

One of the central mechanisms for synapse stabilization is *de novo* protein synthesis. Indeed, *in vivo* blockade of protein synthesis results in synapse disassembly (Bednarek and Caroni, 2011; Caroni et al., 2012). Therefore, the regulation of protein synthesis via multiple kinases and phosphatases might be another major mechanism for local control of synapse assembly and disassembly processes (Caroni et al., 2012). For example, it was shown that mRNA of Arc – a cytosolic protein essential for normal brain function – is enriched at the site of local synaptic activity, implying that Arc protein is locally synthesized (Steward et al., 1998). Indeed, protein kinase A (PKA) and MAPK cascades are required for Arc mRNA induction by Ca^{2+} and cAMP (Waltereit et al., 2001). This finely tuned regulation of mRNA and protein

localization and expression points to highly structured molecular mechanisms ensuring the stabilization of synapses only where they are needed.

Although recent research has given some insight into specific post-translational molecular mechanisms that control synapse stability/disassembly, so far our understanding of this process still remains in its infancy and we know relatively few kinases and phosphatases involved. To understand the complex regulation of synaptic development, function, and stability, novel kinases and phosphatases must be identified with potential roles in these processes.

Drosophila Neuromuscular Junction as a Model System to Study Synapse Stabilization

The precise connectivity necessary for proper circuit function depends on the formation and stabilization of the correct synapses. In mammals, the number of synapses formed at early stages of development largely exceeds the number of synapses retained at later stages, implying that only selective synapses are stabilized and maintained throughout development (Caroni et al., 2012; Goda and Davis, 2003; Luo and O'Leary, 2005). The Drosophila neuromuscular junction (NMJ) is an excellent model system to study synapse development, function, and plasticity. Notably, Drosophila NMJ is glutamatergic and therefore its molecular components and developmental processes are likely to resemble vertebrate central glutamatergic synapses (Jan and Jan, 1976). Despite its stereotyped connectivity, the Drosophila NMJ exhibits developmental and functional plasticity, as synaptic structure and function can be altered by changes in environment, neuronal activity, and/or gene function (Collins and DiAntonio, 2007). The rationale for using Drosophila NMJ as a model system to study synapse formation and stabilization was discussed in detail in my Master Thesis (Bulat 2010). In addition, several outstanding reviews of this system have been published over the last years (Budnik and Ruiz-Canada 2006; Collins and DiAntonio, 2007; Olsen and Keshishian, 2012; Menon et al., 2013).

Although larval NMJ development has been extensively studied (discussed in Master Thesis, Bulat 2010; Menon 2013), not much is known about the molecular mechanisms initiating synapse retraction. Yet the approximate sequence of events that occurs during synapse elimination has been outlined (Eaton et al., 2002; Pielage et al., 2008; Pielage et al., 2005). One of the earliest events during synapse elimination is the retraction of the microtubule cytoskeleton. It has also been demonstrated that presynaptic elimination of synapsin- and vesicle-associated

proteins comes before the removal of postsynaptic receptors. This loss is pictured as a ‘’footprint’’ – one or several boutons that stain for postsynaptic markers that are not opposed by presynaptic structures (Eaton et al., 2002). Importantly, retractions are often confined to specific regions and/or individual branches and do not always result in the complete elimination of the input. Thus, these events can occur locally.

Synapse formation, growth, and maintenance at the Drosophila NMJ are regulated by multiple signaling pathways described in numerous review articles. They include Wingless (Wnt), Bone morphogenic protein (BMP), Transforming growth factor-beta (TGF-beta), Neurexin-Neuroligin, Draper/Ced6, and Synaptotagmin-4 retrograde pathways (Menon 2013) (Chen and Cheng, 2009; Collins and DiAntonio, 2007; Knight et al., 2011; Koles and Budnik, 2012; Marques, 2005). The most extensively studied transsynaptic pathways, such as Wnt, BMP, and TGF-beta, are also discussed in my Master Thesis (Bulat 2010). Although many components and their roles in these signaling pathways were elucidated, surprisingly, to date only a few proteins have been discovered to be directly implicated in NMJ synapse stability. Interestingly, the molecules that are implied in synapse stabilization are also directly or indirectly involved in regulating the cytoskeletal network. These known molecules are the cell adhesion molecule Neuroglian (Enneking 2013, in press), the microtubule-associated protein Stathmin (Graf et al., 2011), the actin-capping molecule Hu-li tai shao (Hts)/Adducin (Pielage et al., 2011), the adaptor molecule Ankyrin 2 (Ank2) (Pielage et al., 2008), the actin-regulator LIM kinase (Eaton and Davis, 2005), the cytoskeletal proteins α- and β-Spectrin (Pielage et al., 2005), βPS integrin (Beumer et al., 2002), the cell adhesion molecule Fasciclin II (Schuster et al., 1996), and the motor protein kinesin (Hurd and Saxton, 1996). Interestingly, it has been shown that the speed at which synapses disassemble is usually faster than the rates of protein turnover (Huh and Wenthold, 1999), indicating that there are mechanisms actively reinforcing synapse disassembly process. Therefore, the identification of additional molecules and regulatory mechanisms locally regulating synapse stabilization/disassembly remains a key interest.

Kinases and Phosphatases in Drosophila NMJ Development and Stability

Protein phosphorylation plays a central role in regulating many aspects of synaptic function (Soderling and Derkach, 2000; Wu et al., 2010). Phosphorylation of synaptic proteins is controlled by kinases and dephosphorylation is controlled by phosphatases. The Drosophila

genome encodes 252 kinases and 86 phosphatases, most of which have mammalian homologues (Morrison et al., 2000). Importantly, there is a high degree of structural and functional conservation of kinases and phosphatases between different organisms (Morrison et al., 2000). Interestingly, the 24 kinase families found in fly are all present in human (Manning et al., 2002).

The importance of kinases and phosphatases in synapse development has been recently elucidated in numerous studies. The kinases and phosphatases implicated in the Drosophila NMJ development include the serine/threonine kinase Leucine-rich Repeat Kinase 2 (LRRK2) (Lee et al., 2010), the proline-directed serine/threonine Cyclin-dependent kinase 5 (Cdk5) (Kissler et al., 2009), the phosphoinositide 3 kinase PI3K and its downstream kinase PKB/Akt (Martin-Pena et al., 2006), a regulatory B` subunit, well-rounded (wrd), of protein phosphatase PP2A and the catalytic subunit of PP2A (Viquez et al., 2009; Viquez et al., 2006), the serine/threonine kinase Shaggy (Franco et al., 2004), a protein kinase C family member aPKC (Ruiz-Canada et al., 2004) and the receptor protein tyrosine phosphatase Lar (Kaufmann et al., 2002). Surprisingly, despite considerable amount of research devoted to studying signaling networks controlling synapse development, to date only one kinase has been discovered to control synapse stabilization at the Drosophila NMJ - serine/threonine kinase Lim kinase1 (LIM), a known regulator of the actin cytoskeleton. LIM functions within the Wnt signaling pathway. It binds to Wnt receptor and is necessary for synaptic stability but is not required for normal synaptic growth or function (Eaton and Davis, 2005). Collectively, these studies suggest that synapse formation and maintenance can be controlled by various kinases and phosphatases.

It is likely that many kinases and phosphatases function in multiple signaling networks. Although several mentioned above studies demonstrated the importance of kinases and phosphatases in the NMJ development, no complete genetic analysis has been performed so far. Therefore, I performed an unbiased RNAi screen of the kinome and phosphatome of Drosophila and identified 7 kinases and 4 phosphatases important for synapse stability: CK2β, Casein kinase Iα (CKIα), Minibrain (Mnb), MAST, 1-phosphatidylinositol 4 kinase (PI4KIIIα), Insulin-receptor kinase (inR), Cyclin-dependent kinase 2 (Cdc2/Cdk1), Protein phosphatase 2A 29B (PP2A-29B), CG9784, Protein phosphatase 4 19C (PP4-19C), and Multiple inositol polyphosphates phosphatase 2 (Mipp2). None of these molecules were previously implicated in synapse stabilization at the Drosophila NMJ. One of the identified kinases is protein kinase CK2.

CK2 General Information

Protein kinase CK2 (formerly Casein Kinase 2) is a serine/threonine kinase consisting of two catalytic (CK2α or CK2α') subunits which associate with a dimer of the non-catalytic (CK2β) subunit. CK2β subunits do not share any homology with other regulatory subunits of protein kinases. CK2 is constitutively active, ubiquitously expressed, can use both ATP and GTP as substrates for phosphorylation, and is found from yeast to man (Blanquet, 2000). The spectrum of actions of CK2 is very diverse, as it can phosphorylate hundreds, if not thousands, of substrates involved in various cellular processes, e.g. cell division, proliferation, cell structure, programmed cell death, DNA repair, translation, gene transcription, and organelle function (Allende and Allende, 1995; Meggio and Pinna, 2003; Pagano et al., 2006). Thus, CK2 is a multifunctional protein kinase with many substrates. Consistent with its many functions, loss of CK2 is lethal in yeast, fly, and mammals (Buchou et al., 2003; Jauch et al., 2002; Lin et al., 2002; Padmanabha et al., 1990).

CK2 Relation to Other Kinases and Evolutionary Conservation

CK2 is a unique protein complex. The catalytic α subunit is only modestly homologous with cyclin-dependent protein kinases, mitogen-activated protein kinases, and Glycogen synthase kinase-3, making CK2 one of the most distant relatives in the protein kinase family (Pinna and Meggio, 1997; Saxena et al., 1987). Nevertheless, the sequences of all CK2 subunits are largely conserved across mammalian and other species. CK2α sequence identity is 90% between Drosophila and human, if the last 53 amino acids lacking in Drosophila are disregarded (Saxena et al., 1987). CK2β is 88% homologous with the human β subunit (Jakobi et al., 1989; Saxena et al., 1987). The α and α' subunits are structurally extremely similar and most of their differences reside in their C-terminal regions (Chen-Wu et al., 1988; Lozeman et al., 1990; Padmanabha et al., 1990). Considering such extraordinary degree of evolutionary conservation, it is reasonable to assume that CK2 might have similar functions in both fly and human.

CK2 Regulation

Although quaternary complex structure is usually a feature of strict regulation, CK2 is a notable exception. For example, PKA holoenzyme, the heterotetrameric structure of which is

closely reminiscent of CK2, is fully inactive unless stimulated by cyclic AMP which leads to the release of active catalytic subunits (Gibson and Taylor, 1997). In contrast, the catalytic subunits of cyclin-dependent kinases (CDKs), which belong to the same group as CK2, are dormant if segregated from a cognate cyclin (Nigg, 1995). The exact mode of regulation of CK2 activity is poorly understood, as none of the conventional regulatory mechanisms are applicable for CK2 (Poole et al., 2005). In contrast to other protein kinases, CK2 possesses at least two autonomous molecular mechanisms to maintain its constitutive activity. First, the activation loop and its N-terminal segment uniquely interact to support constitutive activity of isolated CK2α subunits (Niefind et al., 1998). Second, CK2α subunits associate with the regulatory CK2β subunits, which can reactivate inherently inactive mutants in which the contacts between the N-terminal and activation segments were abolished (Sarno et al., 2002). It is not surprising that CK2 displays oncogenic potential, as its activity is elevated in all tumors examined so far (Munstermann et al., 1990; Piazza et al., 2012; Tawfic et al., 2001).

Although CK2 seems to be spontaneously active toward its substrates, it is generally believed that CK2 activity can be delicately adjusted by phosphorylation, O-GlcNAcylation, ubiquitination, autoinhibitory polymerization, phosphorylation of the β subunit, transient dissociation of α subunits with β dimer, and/or by its association with other proteins (Agostinis et al., 1987; Blanquet, 2000; Guerra et al., 1999; Lolli et al., 2012; Niefind et al., 2001; Olsten et al., 2005; Pinna, 2002; Tarrant et al., 2012). For example, CK2α can autophosphorylate its serine/threonine residues, and this phosphorylation is very weak in the absence of β subunit (Meggio et al., 1983). It was suggested that the role of CK2α autophosphorylation at these residues is to raise its catalytic activity toward CK2 targets (Donella-Deana et al., 2001). Conversely, CK2α autophosphorylation on its tyrosine residue is prevented by association with the β subunit (Donella-Deana et al., 2001). CK2α can also be phosphorylated by the M-phase-specific protein kinase $p34^{cdc2}$ at four residues in its C-terminal, although the consequence of such phosphorylation on CK2 activity is not completely understood (Bosc et al., 1995; Litchfield et al., 1992). It was recently shown that the phosphorylation of at least one of these four residues stabilizes CK2α by enhancing its interaction with Pin1, while O-GlcNAcylation at a neighboring site makes CK2 permissive to proteasomal degradation (Tarrant et al., 2012). Likewise, CK2β can also be phosphorylated at its C-terminus by $p34^{cdc2}$ leading to a significant increase in CK2

activity (Litchfield et al., 1992; Meggio et al., 1995; Mulner-Lorillon et al., 1990). In contrast, autophosphorylation of CK2β in its N-terminus enhances its stability and is correlated with some decrease in CK2 activity (Marin et al., 1997; Pinna and Meggio, 1997; Zhang et al., 2002). In accordance, N-terminal seryl phosphorylation (S2,3,4) of CK2β is important for CK2β function in Drosophila *in vivo* (Jauch et al., 2002). Expressing CK2β constructs with mutations in all three serines (S2,3,4A) in CK2β null mutants rescued the fly viability at a very low frequency (1-5% of the expected number) (Jauch et al., 2002). Thus, although N-terminal seryl phosphorylation does play a role for CK2β function, it is not absolutely indispensable. Although the phosphorylation at its C-terminal might regulate the function of the mammalian CK2, virtually nothing is known about the regulation of Drosophila CK2 via its C-terminus, and neither CK2α nor CK2β most C-terminal region is conserved in Drosophila (Jakobi et al., 1989; Saxena et al., 1987).

Interestingly, a limited number of protein substrates require the presence of β subunit for phosphorylation (e.g. Adrenodoxin), while some other substrates can be phosphorylated only if the β subunits are absent (e.g. calmodulin, PP2A), although those are remarkable exceptions rather than the rule (Arrigoni et al., 2004; Bureik et al., 2005; Heriche et al., 1997; Perez and Avila, 1999). Therefore, free CK2α and/or β subunits might be present in cells as such or arise through transient disassembly of the holoenzyme complex. If that is the case, then the dynamic assembly of the complex might also be a crucial point of its regulation (Filhol et al., 2004; Filhol et al., 2003; Laudet et al., 2008).

Generally, CK2 does not seem to be regulated by any of the well-known second messenger proteins. Although its activity can be subtly modulated by various mechanisms briefly described above, how precisely its activity is controlled is still unclear. Paradoxically, even if there has been considerable amount of research devoted to revealing CK2's mechanisms of regulation, some conflicting views remain as to whether it is constitutively active or activated in response to particular stimuli. One cannot exclude that there may be many distinct sub-populations of CK2 within cells and each of them might be regulated in an individual way.

CK2 Substrates

Although CK2 was discovered approximately 60 years ago (Burnett and Kennedy, 1954), it remains a perplexing enzyme. CK2 participates in a wide array of cellular processes and has a huge variety of potential substrates (Bibby and Litchfield, 2005; Meggio and Pinna, 2003). The vast majority of CK2 targets are implicated in gene expression, protein synthesis, and signaling. The minority of CK2 substrates are metabolic enzymes. Out of 307 CK2 substrates described to date, in only 64 proteins *in vitro* CK2 phosphorylation was not additionally verified by experiments supporting the incidence of *in vivo* phosphorylation (Meggio and Pinna, 2003). The numbers of potential CK2 substrates are constantly increasing and might eventually include a large fraction of the eukaryotic phosphoproteome (Pagano et al., 2006). Indeed, the analysis of available phosphopeptide databases indicates that CK2 alone might account for phosphorylating 10 to 20% of the eukaryotic proteome (Pagano et al., 2006). Such extreme pleiotropism might rationalize why CK2 is constitutively active. Because of these properties, CK2 cannot be compared with the vast majority of protein kinases that perform their functions by being turned on and off at specific steps of signaling pathways. Moreover, unlike most of the serine/threonine kinases which are basophilic enzymes, CK2 recognizes acidic sites.

All residues known to be phosphorylated by CK2 share characteristic phoshphoacceptor sites distinguished by multiple acidic residues (on average more than 5), which surround the phosphoacceptor residue (serine, threonine and, in rare cases, tyrosine). The most important acidic residue is at position n+3 and is found in approximately 90% of the cases. The second most important position is n+1, which is found in approximately 75% of the sites. If the negatively charged determinant is not present at position n+3, it is always present at position n+1 and vice versa (Meggio and Pinna, 2003). Thus, the minimum consensus sequence for CK2 phosphorylation is S-X-X-E/D, although acidic residues can prevail at all positions between n-4 and n+7 (Kuenzel et al., 1987; Marchiori et al., 1988; Marin et al., 1986; Meggio et al., 1984; Meggio and Pinna, 2003). Importantly, basic residues are very rare at CK2 sites and virtually non-existent at positions n+1, n+2, and n+3 (Meggio and Pinna, 2003; Pinna, 2002). Mutational analysis demonstrated that the predisposition of CK2 to interact with negatively charged sites is controlled by distinctive basic residues which reside in the end of the CK2 activation loop, in the glycine rich loop, and in the beginning of helix-C (Sarno et al., 1997). Moreover, the architecture of the active site and the arrangement basic residues implicated in substrate recognition are not substantially amended by association with the β subunit (Niefind et al., 2001).

Although the most prominent feature about CK2 is its pleiotropy, it is absolutely astounding that CK2 still remains a kinase in search of relevant physiological substrates. Due to its unspecific activity profile and its ubiquitous detection in various tissues and cell compartments, the precise functions of CK2 are hard to pin down.

CK2 Substrates in the Nervous System

In the past, it was considered that CK2 represented a kinase mainly required for cell cycle progression in non-neural cells. Thus, a lot of research was devoted to studying CK2 in non-neural tissues. However, recent findings suggest that CK2 has an essential role in the nervous system as well. First, the activity of CK2 is 3-8 fold higher in the brain than in any other tissue. Above all, cortex and hippocampus express especially high levels of CK2 (Blanquet, 2000; Girault et al., 1990; Martin et al., 1990). Second, there seem to be a multitude of substrates in synaptic compartments which have apparent roles in synaptic transmission, plasticity, information storage, and neuritogenesis (Blanquet, 2000). Third, many mouse brain proteins associated with CK2 are relevant to progressive neurological disorders such as neuroblastoma, schizophrenia, Zellweger syndrome, Alzheimer's, Hungtington, and Parkinson's diseases (Aksenova et al., 1991; Iimoto et al., 1990; Perez et al., 2011; Ryu et al., 2008). Fourth, numerous studies implicate a role for CK2 in learning and memory (Blanquet, 2000). Long-term potentiation (LTP) is known to rapidly and transiently increase CK2 activity in hippocampus in a calcium-dependent manner (Charriaut-Marlangue et al., 1991). In addition, many substrates of CK2 are potentially critically significant for LTP establishment and memory consolidation (Blanquet, 2000). Recently, it has been proposed that CK2 plays a prominent role in the induction of LTP through selective regulation of synaptic NMDA receptors (Kimura and Matsuki, 2008). In fact, a recent study by Sanz-Clemente et. al. convincingly demonstrated that indeed, CK2 phosphorylates the NR2B subunit, but not NR2A, to drive NR2B-endocytosis and remove NR2B from synapses (Sanz-Clemente et al., 2010). Thus, CK2 plays an essential role in determining the NR2 subunit composition of synaptic NMDA receptors (Sanz-Clemente et al., 2010). Another notable target of CK2 is the voltage-gated sodium (Na_v) channels, which are highly enriched at the axon initial segment (AIS) and nodes of Ranvier. They play a central role in the generation and propagation of action potentials. It was demonstrated that CK2 plays a crucial role in restricting Na_v channels to the AIS and nodes of Ranvier by phosphorylating

several key serine residues in the AIS-targeting motif of Na_v (Brechet et al., 2008). The phosphorylation enhances its affinity to ankyrin G (ankG), which in turn, is responsible for clustering Na_v channels at the AIS (Brechet et al., 2008). In accordance, the inhibition of CK2 activity resulted in a progressive reduction of Na_v at the AIS (Brachet et al., 2010; Brechet et al., 2008).

CK2 has a role at the postsynaptic side as well. For example, Cheusova et. al. showed that CK2 is accumulates in the subsynaptic muscle membrane and phosphorylates the crucial serine residues within the kinase insert (KI) of the muscle-specific receptor tyrosine kinase (MuSK) (Cheusova et al., 2006). This phosphorylation is essential for normal acetylcholine receptor (AChR) clustering and maintenance by Agrin in cultured myotubes and for the maintenance of synaptic AChR clusters in adult mice (Cheusova et al., 2006). Thus, the study demonstrated the importance of CK2 for the development of subsynaptic specializations. The fact the CK2 phosphorylates such plethora of neuronal substrates suggests that it plays an important role in the nervous system.

CK2α-CK2β Heterotetramer Assembly

CK2β contains a number of conserved structural motifs. Recent biochemical and genetic data clearly indicate that CK2β dimerization is required for the assembly of the CK2 holoenzyme both *in vitro* and *in vivo* and is mediated by the conserved sequence motif CPxxxC-x22-CPxC (Bidwai et al., 1999; Filhol et al., 1991; Graham and Litchfield, 2000; Niefind et al., 2001; Roussou and Draetta, 1994). In fact, the formation of CK2β homodimers through a zinc finger takes place before the incorporation of the catalytic subunits into CK2 complexes (Graham and Litchfield, 2000). In particular, residues within the N-terminal domain of CK2β are sufficient for CK2β subunits interactions, while the C-terminal domain of CK2β is essential for complex formation with the catalytic subunits of CK2 (Graham and Litchfield, 2000). The experiments using the yeast two-hybrid system also suggested that β subunits can interact with both α and β, while CK2α subunits can interact exclusively with β subunits but not with each other (Boldyreff et al., 1996; Gietz et al., 1995; Kusk et al., 1995).

For a long time CK2 was believed to exist as an obligate tetrameric complex *in vivo*, mainly because of the stable nature of the tetramers *in vitro* (Pinna and Meggio, 1997). However,

the traditional view was challenged by X-ray crystallography studies suggesting that CK2 is a transient non-obligatory heterocomplex, as the CK2α/CK2β interface is relatively small (832A^2) and flexible compared to the average interface size (1722A^2) for the permanent subunit association (Jones and Thornton, 1996; Niefind et al., 2001). In accordance, there is some evidence that the unbound α monomers and β dimers can exist *in vivo* under specific conditions and/or in certain compartments for specific functional and regulatory reasons (Filhol et al., 2003). Moreover, unbalanced expression of catalytic and regulatory subunits was demonstrated in a variety of tissues and tumors (Filhol et al., 2004; Guerra et al., 1999; Li et al., 2006). In all probability, the isolated subunits may arise from a spatial or temporal overload of one of the subunits after translation. On the other hand, the subunits could be released by dissociation from the CK2 holoenzyme. However, to this day it is not clear whether the isolated α and/or β subunits are present in cells *in vivo*. In fact, a number of studies imply that α subunit can exist and function alone in cells in the absence of β, while β is often degraded if it is not able to associate with α (Canton et al., 2001; Luscher and Litchfield, 1994; Stigare et al., 1993; Zhang et al., 2002). It is also interesting that only CK2α, but not the holoenzyme, is able to associate with PP2A and increase PP2A's activity (Heriche et al., 1997; Perez and Avila, 1999). On the other hand, some other studies raise the prospect that CK2β can have CK2-independent function in cells (Bibby and Litchfield, 2005). For example, it was observed that CK2β has the potential to regulate other protein kinases, such as c-Mos and A-Raf, which are important for cell proliferation (Boldyreff and Issinger, 1997; Chen et al., 1997). In Drosophila, it is not known whether α or β subunits can transiently exist outside the CK2 complex. It is known, however, that CK2α interacts strongly and equally well with all CK2β isoforms (Jauch et al., 2006). Moreover, mutation of either cysteinyl residue pair, involved in the zinc finger mediated dimer formation and thus association with α subunits, results in a CK2β protein unable to rescue the lethality of the CK2β loss-of-function mutant (Jauch et al., 2002). Therefore, β/β dimerization and consequent association with CK2α subunits appear to be crucial for the *in vivo* CK2β function. The failure of CK2β cysteine mutants to substitute for the loss of endogenous CK2β could be due to the misregulation of CK2α *in vivo* or due to the fast degradation of CK2β mutated protein unable to incorporate into stable CK2 complexes. In fact, the latter was observed in human cysteine CK2β mutants in COS-7 cells (Canton et al., 2001).

Therefore, it would be of great interest to learn more about the role of α-β interaction. We have the possibility to address some of our questions by introducing mutations in CK2α, interfering with its ability to form complexes with the β-dimers (Raaf et al., 2011). Using isothermal titration calorimetry, Raaf et al. identified two conserved residues in human CK2α, L41 and F54 (L39 and F52 in Drosphila), required for binding to CK2β. Mutations in these two amino acids were disruptive only for the association with CK2β but did not perturb CK2α kinase activity (Raaf et al., 2011).

Genetic Manipulation of CK2 in Mammals

CK2α has an essential role for mouse embryonic development. CK2α knockout mice die in mid-embryogenesis (e12.5) with neural tube and cardiac defects (Seldin et al., 2008). Likewise, disruption of CK2β in mice is embryonic lethal. The CK2β mutants display signs of reduced cell proliferation and die at e7.5 (Buchou et al., 2003). Furthermore, the failure to obtain homozygous CK2β knockout embryonic stem cell line strongly implies that CK2β is indispensable for viability at the cellular level (Buchou et al., 2003). These findings suggest that CK2β is either needed for the appropriate modulation of CK2 activity required for survival, or CK2β is essential for performing functions independent of CK2 (or both). Indeed, CK2β appears to be necessary for mediating CK2α activity in the muscle, as muscle-specific CK2β knockout mice have elevated CK2α activity in all muscles examined (Cheusova et al., 2006). As a result, those mice have impaired muscle endplate structure and function and develop a muscle weakness phenotype (Cheusova et al., 2006).

The role of CK2β in the central nervous system was also investigated. Ablation of CK2β in embryonic neural stem cells caused severe telencephalon defects during late development and compromised forebrain progenitor cell proliferation, implying that CK2β is crucial for the proliferation of neural stem cells *in vivo* (Huillard et al., 2010). Unfortunately, due to early lethality, nothing specific is known about the roles of CK2α and CK2β in the nervous system development.

Disruption of the CK2α' gene was also performed in mice. In contrast to CK2α and CK2β, CK2α' knockout mice are viable, however, the males are infertile due to a defect in

spermatogenesis (Escalier et al., 2003; Xu et al., 1999). Therefore, although CK2α' has an essential role is sperm progenitors, CK2α seems to compensate for the loss of CK2α' for maintaining viability. All these data confirm the generally accepted notion that CK2 is a vital enzyme which plays an important role in cell proliferation and embryogenesis.

Genetic Manipulation of CK2 in Drosophila

CK2α: Tik and TikR Mutants. Role in Circadian Rhythms and Neurogenesis

A distinctive feature of Drosophila is the absence of the α' subunit (Allende and Allende, 1995). As in the mouse models, CK2α or β mutations in Drosophila lead to early lethality, substantiating the indispensable role of CK2 for cell and organism vitality. Strong loss-of-function alleles of Drosophila CK2α, named Timekeeper (Tik) and Timekeeper-Revertant (TikR), were discovered in an EMS screen for modifiers of the circadian clock and are both homozygous lethal (Lin et al., 2002).

Animals heterozygous for Tik display lengthened circadian rhythms, implying that this allele acts in a dominant manner (Lin et al., 2002; Smith et al., 2008). *In vivo* biochemical measurements show that Tik mutants lose the CK2 enzymatic function (Lin et al., 2002). Molecular analysis demonstrates that there are two sequence changes in the Tik mutant: M161K and E165D. Of these two residues, M161 is highly conserved in all so far published CK2α sequences from other species and sits within the hydrophobic ATP-binding pocket (Niefind et al., 1998). Presumably, the exchange from hydrophobic (M) to hydrophilic (K) amino acid inhibits ATP-binding and therefore disrupts CK2 kinase activity. Indeed, this assumption was confirmed for a Tik variant of human CK2α (Rasmussen et al., 2005). In contrast, the second mutation which is a conservative change from E165 to D is not expected to have any impact on the kinase activity of the Tik mutant. This assumption is strengthened by the fact that an aspartic acid residue is present in the wildtype CK2α sequence of another insect species, Spodoptera frugiperda (Rasmussen et al., 2005). However, this assumption has not been tested so far and the contribution of the E165D change to the dominant circadian phenotype of Tik animals remains unclear.

In contrast, TikR displays a key genetic characteristic of a revertant, as animals heterozygous for TikR allele do not display a circadian phenotype (Lin et al., 2002). In addition to the two original Tik coding changes, TikR allele contains an additional deletion of 7 amino acids (234-240) and another amino acid substitution (R242E) (Lin et al., 2002). As a result, TikR does not display any kinase activity and is also homozygous lethal. Because the CK2α TikR protein is much less soluble, it was suggested that folding of CK2α TikR is changed *in vivo*, impairing association with CK2β and formation of the holoenzyme. Although this possibility was not tested, this might be the reason why TikR/+ flies do not display severe clock defects (Lin et al., 2002).

In addition to the role of CK2 in circadian rhythms, the functional dissection of Tik mutation implicated a role for CK2 during Drosophila neurogenesis, a process that leads to the stereotyped patterning of sensory organs, bristles, and eye (Kunttas-Tatli et al., 2009).

CK2α: H3091 and G703 Mutants. Role in Eye Development

Three studies implicate a potential role for CK2 during eye development. CK2α H3091 and G703 alleles were identified in a forward genetic screen selecting for visual system wiring defects. Both are single substitutions of two conserved residues in the C-terminal part of CK2α gene (D212N and W279G respectively), leading to the medulla targeting defects (Berger et al., 2008). A potential role for CK2α during retinal patterning was identified by Karandikar et. al. The researchers implicated CK2 as a regulator of E(spl) and generated a variant of E(spl)M8 that replaced S159 with the phosphomimetic amino acid D. They found that M8SD dominantly interfered with eye development (Karandikar et al., 2004). In addition, Bose et. al. compromised CK2α functions by UAS-RNAi against CK2α or by UAS-Tik which behaves in a dominant-negative manner. Compromising CK2 function by either approach led to neuronal defects in the eye and bristle, which are similar to those after the loss of E(spl) (Bose et al., 2006). Therefore, CK2α is required for normal eye morphogenesis and medulla targeting.

CK2β: Mbu^{P1} and Mbu^{26-2} Mutants. Role in Development and Cell Proliferation/Survival

Similarly, null and hypomorphic alleles were identified for CK2β. In Drosophila, there are three CK2β subunits: CK2β, CK2β', and CK2βtes. Both CK2β' and CK2βtes are testis-

specific and CK2β is expressed ubiquitously (Kalmykova et al., 1997; Kalmykova et al., 2002). CK2β transcription unit encodes for several isoforms different in their C-terminal, which can influence their functional properties (Jauch et al., 2006).

A CK2β allele characterized by two P-element insertions into the first non-coding CK2β exon, named mushroom bodies undersized (mbu^{P1}), was identified in a genetic screen for mutations affecting the structural organization of the central brain (Jauch et al., 2002). CK2β mbu^{P1} allele causes a reduced expression of all identified CK2β isoforms, which is in good agreement with the notion that the P-element insertion into CK2β leads to the decreased amounts of the primary CK2β transcript (Jauch et al., 2006). The phenotype of this viable mutation points to a function for CK2β in cell proliferation and/or survival during brain development. In mbu^{P1} flies, there is a great reduction in the number of neurons (Kenyon cells) in the adult mushroom bodies, which is a structure engaged in memory processes (Heisenberg, 1998; Jauch et al., 2002). A complete loss-of-function allele CK2β Mbu^{26-2} resulted from an imprecise excision of this P-element. This deletion removes a genomic segment that is 3' of the original mbu^{P1} insertion and includes 5' untranslated region and coding sequences of CK2β (Jauch et al., 2002). Animals homozygous for CK2β die as embryos or early first instar larva (this study), thus, confirming an indispensable role of CK2β in development.

CK2β: Andante Mutation. Role in Circadian Rhythms

CK2β was also identified as a molecular clock protein in Drosophila. The hypomorphic allele Andante (M166I) affects the circadian rhythms and mushroom body development (Akten et al., 2003; Jauch et al., 2006). Interestingly, this conservative amino acid change in Andante mutant leads to the reduced levels of three CK2β isoforms out of five, the reason for which is unclear (Jauch et al., 2006). The Andante mutation might cause a defect in CK2β dimerization and/or α2- β2 holoenzyme formation, as some studies show that both Drosophila and human β subunits are unstable and subjected to degradation in the absence of the α subunit (Bidwai et al., 1999; Luscher and Litchfield, 1994). However, this assumption is not supported by the by the structural data according to which CK2β dimerization proceeds mostly due to a zinc finger domain (Chantalat et al., 1999). This domain is not in the proximity of the Andante mutation.

Moreover, a single conservative amino acid substitution is extremely unlikely to disturb the interaction with the CK2α subunits, as can be determined from the mapped interaction sites of the three-dimensional structure of the CK2 holoenzyme (Niefind et al., 2001). Gel filtration assays with wild type CK2β and Andante-CK2β support the notion that M166I mutation does not influence the β/β and β/α assembly (Rasmussen et al., 2005). Therefore, the reason for the reduced levels of β subunit in Andante mutants remains unclear. In addition, animals homozygous for Andante do not show any major developmental defects (Akten et al., 2003).

Interestingly, although quite a few CK2α and CK2β mutants are available, not much is known about the CK2 function in the nervous system. Moreover, absolutely nothing is known about the potential role of CK2 at the Drosophila NMJ.

Aim of the study

The aim of this study was the unbiased identification of signaling pathways controlling synapse stabilization *in vivo* by performing a high-resolution RNA interference (RNAi) based screen. I then focused my analysis on the role of Casein Kinase 2 (CK2) for synapse maintenance.

Manuscript

Casein Kinase 2 Controls Synapse Organization and Stability

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Running title: CK2 controls synapse stability

Summary

Structural synaptic plasticity, the regulated assembly and disassembly of functional synaptic connections, is essential for the development and adjustment of neuronal circuits. However, the regulatory mechanisms enabling local control of synapse formation, stabilization and elimination remain largely unknown. Using an *in vivo* RNAi-based screen targeting the kinome and phosphotome of Drosophila we identified 11 kinases and phosphatases that control synapse stability through modulation of phospholipid signaling, metabolic signaling or the cytoskeleton. We focus our analysis on Casein kinase 2 (CK2) and demonstrate that both the regulatory CKβ-subunit and kinase activity mediated by the catalytic CK2α-subunit are essential for synapse maintenance. In addition, CK2 activity controls the organization of the presynaptic nerve terminal into distinct synaptic vesicle domains. CK2α and CK2β mutually depend on each other and we identified essential residues in CK2α that mediate this interaction and are essential for synapse stability *in vivo* demonstrating that CK2 functions as an α2/β2 holoenzyme. We identified Ankyrin2, an adaptor molecule linking synaptic cell adhesion molecules to the cytoskeleton, as an essential target of CK2. Control of synapse stability via constitutive phosphorylation of structural synaptic components provides a compelling mechanism to regulate the lifetime of synaptic connections and to enable local structural synaptic plasticity without disruption of neuronal circuit architecture.

Introduction

Information processing in the brain relies on the precise formation of neuronal circuits. These networks are required for the integration of sensory and motor information and for cognitive functions including learning and memory. Recent work demonstrates that adaptations in neuronal processing are not only mediated by Hebbian modulation of synaptic function but also by structural remodeling of neuronal networks through the selective assembly of disassembly of identified synaptic connections (Holtmaat and Svoboda, 2009; Xu et al., 2009; Yang et al., 2009). Live-imaging studies in the mouse cortex demonstrated a steady-state turnover of synapses at any time that is significantly higher during initial development of neuronal circuits (Holtmaat and Svoboda, 2009). Sensory stimulation or motor learning paradigms cause a significant increase in structural synaptic plasticity to adjust information

processing and to enable storage of information within the circuit (Xu et al., 2009; Yang et al., 2009). These activity-dependent processes facilitate the formation of new synaptic connections in close proximity to existing stable synapses thereby enabling strengthening selective neuronal connectivity (De Roo et al., 2008; Fu et al., 2012; Harvey et al., 2008). The same stimuli also cause the selective elimination of previously functional stable synapses. These studies demonstrate that neuronal connectivity is controlled with high accuracy in a local and temporal precise manner and by regulated formation, stabilization and elimination of synapses. In contrast, any inappropriate loss of synaptic connections due to genetic perturbations will result in an impairment of information processing and is a likely cause of mental impairment in psychiatric and neurodegenerative diseases (Lin and Koleske, 2010). Despite the importance of local regulation of structural plasticity the molecular mechanisms underlying these phenomena remain largely elusive. Likely mechanisms include local control of protein synthesis or stimulation of signaling cascades impinging on the regulation of the pre- and postsynaptic cytoskeleton and trans-synaptic cell adhesion (Caroni et al., 2012; Saneyoshi et al., 2011). Posttranslational modifications including phosphorylation have been demonstrated to locally control synaptic cell adhesion molecules and to activate regulators of the actin cytoskeleton (Benson and Huntley, 2012; Brigidi and Bamji, 2011; Saneyoshi et al., 2011; Siddiqui and Craig, 2011). Indeed local regulation by kinases and phosphatases is important for multiple aspects of synapse development and function including mechanisms underlying learning and memory (Mayford, 2007; Siddiqui and Craig, 2011).

Here, we perform a high-resolution *in vivo* RNA interference (RNAi) based screen to systematically identify kinases and phosphatases controlling synapses stability. The Drosophila neuromuscular junction (NMJ) represents an ideal model system as synapse stability can be analyzed at the resolution of individual synapses. This system allowed the identification of regulators relevant for structural synaptic plasticity underlying learning and memory (Bednarek and Caroni, 2011; Pielage et al., 2011; Ruediger et al., 2011) and of potential neurodegenerative disease genes in the past (Eaton et al., 2002; Hafezparast et al., 2003; Ikeda et al., 2006; Jenkins et al., 2001; Koch et al., 2008; Pielage et al., 2008; Pielage et al., 2006). We selectively targeted more than 90 % of all kinases and phosphatases encoded in the Drosophila genome. The knock down of 7 kinases and 4 phosphatases resulted in a significant increase in synapse retractions. We identified metabolic signaling, phospholipid modifications and regulation of the cytoskeleton

as core pathways regulating synapse stability. We then focused our analysis on the role of casein kinase 2 (CK2) for synapse maintenance.

CK2 is a highly conserved serine-threonine kinase that is composed of two catalytic (CK2α) and two regulatory (CK2β) subunits that together form a heterotetrameric α2β2 holoenzyme. CK2 is ubiquitously expressed, constitutively active and phosphorylates serine or threonine residues in an acidic environment (Allende and Allende, 1995; Meggio and Pinna, 2003). Hundreds if not thousands of potential targets controlling diverse cellular process have been identified to data (Meggio and Pinna, 2003; Salvi et al., 2009). While no clear activation mode has been identified so far, CK2 activity can be modulated by expression, phosphorylation and potentially interaction with the regulatory subunit. CK2α interacts with a preformed dimer of CK2β subunits that has been implied as important for the stability of the complex, modulation of activity and target interaction (Graham and Litchfield, 2000; Niefind et al., 2001). Due to the stability of the complex it has been suggested that cellular CK2 activity might almost exclusively be due to the α2β2 holoenzyme. However, isolated subunits can exist *in vivo* and CK2α has the potential to phosphorylate target proteins in the absence of CK2β (Heriche et al., 1997; Marin et al., 1999; Martel et al., 2002; Meggio et al., 1992; Pinna and Meggio, 1997; Salvi et al., 2006).

CK2 has been implicated as an essential regulator of neuronal development and function based high expression levels in brain especially in centers implicated in learning and memory including the hippocampus and cortex (Blanquet, 2000). Long-term potentiation (LTO) rapidly and transiently increases CK2 activity in the hippocampus (Charriaut-Marlangue et al., 1991). Consistent with an essential function during neuronal development mice mutant for the catalytic CK2α subunit die in mid-gestation (E11.5) with structural defects in heart and neural tube (Lou et al., 2008; Seldin et al., 2008). Similarly, CK2β knock out mice die at E7.5 (Buchou et al., 2003) and disruption of CK2β in embryonic neural stem cells causes severe defects in the telencephalon during development due to defects in neural stem cell proliferation (Huillard et al., 2010). An alternative catalytic subunit CK2α' is only essential in the male germ line (Xu et al., 1999). The severe developmental phenotypes prevented a detailed analysis of the function of CK2 for neuronal and synaptic development. Using culture systems and CK2 specific inhibitors it has been demonstrated that CK2 can phosphorylate the cell adhesion molecule L1 CAM (Wong et al., 1996) and is required for the activity dependent switch from N-methyl-D-aspartate

receptor (NMDAR) NR2B to NR2A during development of cortical synapses (Chung et al., 2004; Sanz-Clemente et al., 2010). In addition, CK2 restricts voltage-gated sodium (Nav) channel distribution to the axon initial segment (AIS) and nodes of Ranvier by regulating the interaction with the adaptor molecule Ankyrin G (AnkG) (Brachet et al., 2010; Brechet et al., 2008). A conditional muscle specific knock out demonstrated that CK2β is required for the phosphorylation of MuSK for maintenance of synaptic acetylcholine receptors clusters (Cheusova et al., 2006).

The Drosophila genome encodes a catalytic CK2α subunit, a regulatory subunit CK2β and two alternative testis-specific CK2β subunits (CK2β and CK2βtes) but lacks an alternative catalytic CK2α' subunit (Allende and Allende, 1995; Kalmykova et al., 1997; Kalmykova et al., 2002). CK2α and CKIIβ are essential for Drosophila development and null mutations die at early larval stages (Jauch et al., 2002; Lin et al., 2002) (this study). Using dominant-negative and hypomorphic mutations CK2 has been implicated in diverse processes including circadian clock regulation, photoreceptor targeting and brain development (Akten et al., 2003; Berger et al., 2008; Jauch et al., 2002; Jauch et al., 2006; Lin et al., 2002).

Here, we analyze the contribution of CK2α and CK2β for synapse development and maintenance using loss-of-function mutations. We demonstrate that both CK2α and CK2β are essential for synapse stability and that CK2α kinase activity is essential for both synapse maintenance and organization of the presynaptic nerve terminal. We provide biochemical and *in vivo* evidence that CK2 functions as an α/β holoenzyme *in vivo* and identify essential CK2α residues mediating this interaction in vivo. We then identify the adaptor molecule Ankyrin2 as a target for CK2 and provide evidence that CK2-dependent regulation of Ankyrin2 is essential for synapse maintenance.

Results

Goal of this study was the unbiased identification of signaling modules controlling synapse maintenance *in vivo.*

RNAi screen identifies kinases and phosphatases essential for synapse stability

To identify the regulatory mechanisms controlling the maintenance of synaptic connections we performed an *in vivo* RNA-interference (RNAi) based screen of the Drosophila

kinome and phosphatome. At the Drosophila NMJ, synapse stability can be assessed at the resolution of individual synapses by monitoring the apposition of pre- and postsynaptic markers. In wild type animals the presynaptic active zone marker Bruchpilot (Brp) is always found in precise opposition to postsynaptic glutamate receptor clusters. Synaptic retractions as indicated by a loss of Brp despite persisting glutamate receptor clusters, occur only at distal boutons at very low frequency (≤ 5% of all NMJs). In contrast, knock down or mutations of genes essential for synaptic maintenance will result in a significant increase in the frequency and severity of synaptic retractions and can lead to the complete elimination of the presynaptic nerve terminal (Eaton et al., 2002; Pielage et al., 2011; Pielage et al., 2008; Pielage et al., 2005). Thus, this *in vivo* assay allows a systematic and unbiased identification of novel regulators of synapse stability.

We identified 476 transgenic RNAi-lines (VDRC RNAi-collection) (Dietzl et al., 2007) targeting 312 kinases and 127 phosphatases and their regulatory subunits thereby representing more than 90% of the entire Drosophila kinome and phosphatome (Supplemental table 1). We expressed transgenic dsRNA constructs in presynaptic motoneurons and monitored potential defects in synapse stability using our high-resolution assay. The knock down of 11 kinases and phosphatases resulted in a significant increase in synapse retractions, as indicated by a loss of presynaptic Brp and a fragmentation of the presynaptic membrane despite the presence of postsynaptic glutamate receptor clusters (Figure 1). We verified these results using independent dsRNA lines (VDRC, Vienna or TRiP, Bloomington) for four candidates demonstrating that the observed effects were due to a specific knock down of the target gene (Figure 1 G; Supplemental Figure 1 G, Supplemental table 2). We observed a large variance regarding the frequency and severity of synaptic retractions between different target genes and dsRNA lines (Figure 1 G, H). Synaptic retractions were evident at rates ranging between 7 to 50% of analyzed NMJs within animals. The severity of synaptic retractions varied ranging between absence of presynaptic markers and membrane fragmentation only at distal boutons (Figure 1E; Supplemental Figure 1F) to entire NMJ branches (Figure 1C, D, F; Supplemental Figure 1 B, C, E) and to complete eliminations of the entire presynaptic nerve terminal (Figure 1 B; Supplemental Figure 1D). We analyzed synaptic retractions on ventral, medial and dorsal muscle groups and observed significant differences in synaptic retraction frequencies for different target genes at these muscles (Figure 1 G; Supplemental Figure 1 G; Supplemental table 2). To ensure that the

observed phenotypes represented de facto synaptic retractions and not only aberrant localizations of presynaptic Brp, we analyzed all genotypes with the presynaptic vesicle marker Synapsin (Syn) and the postsynaptic marker Discs-large (Dlg). For all genotypes we observed qualitative and quantitative similar phenotypes (Supplemental Figure 2). Thus our screen successfully identified kinases and phosphatases that are required within the presynaptic motoneuron to control the maintenance of synaptic connections. To elucidate the cellular mechanisms underlying regulation of synapse stability we focused our analysis on the β-subunit of Casein Kinase 2 (CK2β), as knock down of *CK2β* resulted in one of the most severe phenotypes identified in our screen (Figure 1G).

Presynaptic CK2β is essential for synapse stability

We first tested whether CK2β is required only pre- or also postsynaptically for synapse maintenance. Knock down in the presynaptic motoneuron resulted in synaptic retractions at up to 50% of NMJs depending on the muscle group analyzed (Figure 2 B, G; S-Table 2). The analysis of retraction severity demonstrated that the presynaptic disassembly often includes more than 7 postsynaptic profiles (boutons) that are no longer opposed by Brp (Figure 2 H). In contrast, knock down in the postsynaptic muscle did not significantly increase retraction frequency or severity compared to control animals (Figure 2 C, G, H). To control for the specificity of our RNAi-mediated knock down we generated a specific antibody against CK2β. This antibody specifically recognizes CK2β on Western blots of larval brain extracts but does not recognize CK2β in situ. We observed an efficient knock down of CK2β protein levels in larval brain extracts using neuronal but not using muscle specific expression of CK2β RNAi (Figure 2 K). To further demonstrate that the observed effects on synapse stability are due to CK2β knock down we generated transgenic flies enabling expression of wild type CK2β under UAS-control. Co-expression of CK2β but not of membrane-bound GFP (mCD8-GFP) in motoneurons was able to restore synaptic stability (Figure 2 G) and to restore CK2β protein levels (Figure 2K). Next, we aimed to complement the RNAi-based results by analyzing mutations in *CK2β*. Mutations in *CK2β* (also known as *mushroom body undersized*, *mbu*) were previously identified based on their function for mushroom body development and for the regulation of circadian rhythm (Akten et al., 2003; Jauch et al., 2002). While the hypomorphic $CK2\beta^{P1}$ (mbu^{P1}) mutation is

adult viable, the null allele *CK2β*$^{26\text{-}2}$ (*mbu*$^{26\text{-}2}$) results in lethality at the first/second instar larval stage. Transheterozygous *CK2β*^{P1}/ *CK2β*$^{26\text{-}2}$ mutant larvae showed a small but significant increase in synaptic retractions (Figure 2 G, H). The quantitative differences compared to the RNAi evoked phentoypes are likely due a lesser effect on neuronal CK2β protein levels (Figure 2 K). The weaker nature of this allelic combination is underscored by the fact that transheterozygous *CK2β*^{P1}/ *CK2β*$^{26\text{-}2}$ animals were viable while neuronal experession of *CK2β* -RNAi led to pupal lethality. To directly analyze motoneurons lacking all CK2β protein, we used the MARCM technique to generate *CK2β* null mutant motoneurons that were positively marked by membrane-bound GFP (Lee and Luo, 1999). Control clones did not show any impairment in synapse stability as indicated by the perfect opposition of presynaptic Brp and postsynaptic GluRIII at all NMJs (Figure 2 D, I, J). In contrast, motoneurons lacking *CK2β (CK2β*$^{26\text{-}2}$ MARCM) resulted in synaptic retractions at 33% of mCD8-GFP-marked NMJs. This rate is consistent with our RNAi-based results as we scored NMJs on all muscles with no bias to a certain muscle group (S-Table 2). Finally, we were able to rescue the lethality and synaptic retractions and to restore CK2β protein levels of *CK2β*$^{26\text{-}2}$ null mutant animals by ubiquitous expression of *CK2β* (Figure 2, G, K). Together our data demonstrates that presynaptic CK2β is essential for the maintenance of synaptic connections.

Presynaptic CK2α is essential for synapse stability

CK2β encodes the regulatory subunit of the CK2 holoenzyme that is composed of two catalytic CK2α and two CK2β subunits (α2/β2). To address potential unique or shared functions of the two subunits and of the CK2 holoenzyme we next analyzed the requirements of CK2α for synapse stability. Mutations in CK2α were previously identified in genetic screens for regulators of circadian rhythm (CK2α^{Tik} disrupts circadian rhythm in a dominant manner; CK2α^{TikR} is a revertant of CK2α^{Tik} and likely null allele) and for axon guidance in the eye (CK2α^{H3091} and CK2α^{G703}) (Figure S3A) (Akten et al., 2003; Berger et al., 2008; Jauch et al., 2002; Lin et al., 2002). However, the consequences of strong loss-of-function mutations for nervous system or synapse development have not been analyzed so far. Here, we identified two Piggybac insertions (PBac_LL05896, referred to as CK2α^{P1} and PBac_LL07221 referred to as CK2α^{P2}; splice-acceptor mutations, generated in (Schuldiner et al., 2008); www.flybase.org) as potential alleles

of *CK2α* (Figure S3 A). Complementation analyses demonstrated that all mutations, including the piggyback insertions CK2α^{P1} and CK2α^{P2}, represent alleles of *CK2α*. While homozygous and transheterozygous P2 and TikR mutations resulted in early first instar larval lethality, animals homozygous for P1 or transheterozygous combinations of all other alleles survived at least to the third instar larval stage. Based on their lethality phase we could place the mutations into the following allelic series (CK2α^{P2} = CK2α^{TikR} > CK2α^{P1} ≥ CK2α^{Tik} > CK2α^{H3091} ≥ CK2α^{G703}; Supplemental Table 2). These results correlated well with the molecular nature of the different alleles (Figure S3). Importantly, all transheterozygous and homozygous CK2α^{P1} mutations caused a significant increase in the frequency of synaptic retractions compared to control animals (Figure 3A-C; Supplemental Figure 4A, B). We observed the highest synaptic retraction frequencies for allelic combinations of strong hypomorphic alleles that still survived to late larval stages (Figure 3 G, Supplemental Figure 4A, B, Supplemental table 2). We observed slightly weaker phenotypes on ventral muscle groups (muscles 6, 7) compared to dorsal muscle groups (muscles 1, 2, 9, 10), however, the relative phenotypic strength of the different mutations was identical (Supplemental Figure 4A, B; Supplemental Table 2). To analyze potential tissue-specific functions of CK2α we generated a UAS construct allowing directed expression of wild type CK2α. In addition, we generated a kinase-dead version of CK2α (*CK2α^{KD}*) by mutating the lysine 66 to methionine (K66M) thereby mimicking the *in vitro* characterized kinase-dead mutation of human CK2α (Figure 4B) (Penner et al., 1997). This allows us to directly assess the requirements of kinase-activity of CK2α. We used phi-c31-mediated site-specific integration into the *attP40*-landing site for all transgenic constructs in this study to ensure equal expression levels (Bischof et al., 2007). To monitor expression levels directly we generated a CK2α-specific antibody. We were able to rescue the lethality of all transheterozygous combinations and of homozygous *CK2α^{P1}* and *CK2α^{P2}* mutant animals by ubiquitous expression of CK2α (*da*-Gal4) demonstrating the specificity of all mutations (Figure 3 G; Supplemental Table 2 and data not shown). Importantly, pre- but not postsynaptic expression of CK2α was sufficient to restore synapse stability and to restore neuronal CK2α protein levels in all analyzed combinations (Figure 3 D, E, G, H). Due to proximity of CK2α to the centromere we were unable to analyze the strong loss of function mutation *CK2α^{P2}* using MARCM clones. To address this issue, we rescued the lethality of *CK2α^{P2}* mutants by ubiquitous expression of *CK2α* and, at the same

time, prevented neuronal expression (*da*-Gal4; *elav*-Gal80). We observed synaptic retractions at 43% of analyzed NMJs in these animals which is in accordance with our analysis of trans-heterozgous combinations (Figure 3 G). Importantly, CK2α kinase activity is essential for synapse stability as presynaptic expression of $CK2\alpha^{KD}$ was not sufficient to rescue synaptic retractions despite being expressed at similar levels as wild type CK2α (Figure 3F, G, H). In addition, ubiquitous expression of $CK2\alpha^{KD}$ did not restore viability of any *CK2α* mutation. Thus, we conclude that presynaptic CK2α kinase activity is required to maintain the presynaptic nerve terminal.

Presynaptic CK2α controls organization of synaptic domains

We next asked whether CK2α is required for additional aspects of synapse development. Analysis of NMJ morphology revealed a significant, 30% decrease of synaptic bouton number in CK2α mutant animals compared to wild type controls (Figure 4 E). In addition, analysis of synaptic vesicle domains within presynaptic boutons revealed a requirement of CK2α for subcellular organization of the presynaptic nerve terminal. In contrast to wild type, synaptic vesicle clusters in *CK2α* mutants are not organized into distinct clusters within individual boutons but appear fused (Figure 4 A, B). The quantification demonstrated a significant, more than 60-percent reduction in the number of individual DvGlut-positive synaptic vesicle clusters in *CK2α* mutant animals compared to wild type (Figure 4 F). This reduction in cluster number was still significant when taken into account the general reduction in NMJ size in these animals and was clearly apparent when analyzing individual synaptic boutons (Figure 4 A, B). Importantly, presynaptic expression of wild type CK2α in *CK2α* mutants was sufficient to fully rescue bouton number and subcellular synaptic vesicle domain organization (Figure 4 C, E, F). In contrast, expression of $CK2\alpha^{KD}$ failed to restore NMJ organization or growth demonstrating the necessity of CK2α kinase activity (Figure 4 D, E, F). We next asked whether CK2α is also sufficient to control presynaptic organization in a wild type background. As CK2α activity correlates with CK2α protein levels in other systems (Trembley et al., 2009) we expressed high levels of CK2α in motoneurons (2 copies elav-Gal4> 2 copies UAS-*CK2α*). Interestingly, in these animals, we observed the opposite phenotype of the CK2α loss-of-function phenotype. Instead of fewer synaptic boutons and large fused clusters of presynaptic vesicles the NMJ

became subdivided into smaller individual domains. We observed a highly-significant, more than 30-fold, increase in the number of satellite boutons compared to controls (control 0.6±0.19, high levels CK2α 20.45±2.44 satellite boutons/ muscle 4 NMJ; n = 20 NMJs, 5 animals). Individual synaptic vesicle domains were smaller, more abundant and clearly separated within the presynaptic nerve terminal (Figure 4 G). Analysis with pre- and postsynaptic markers revealed that synapses were no longer confined to large synaptic boutons but were surrounded by their own synaptic vesicle domain and separated within the presynaptic nerve terminal (Figure 4 G, I). The quantification demonstrated a significant, 1.7-fold, increase in the number of synaptic vesicle cluster number in CK2α overexpressing animals compared to controls (Figure 4 F). In contrast, expression of equally high levels of $CK2\alpha^{KD}$ did not cause any obvious NMJ phenotypes (data not shown) demonstrating that presynaptic CK2α kinase activity is necessary and sufficient to control subcellular organization and stability of the presynaptic nerve terminal.

CK2α – CK2β interaction is essential for the control of synapse stability

We next addressed potential mechanisms controlling CK2 function *in vivo*. *In vitro* CK2α has the ability to phosphorylate CK2 targets independent of CK2β, however it has been suggested that CK2 functions primarily as a α2β2 holoenzyme *in vivo* (Heriche et al., 1997; Litchfield, 2003; Marin et al., 1999; Meggio and Pinna, 2003). In support of this hypothesis we observed a reduction of CK2β protein levels in larval brain extracts of CK2α mutations that reduce CK2α protein levels ($CK2\alpha^{P1}$, $CK2\alpha^{P2}$, $CK2\alpha^{TikR}$) but not in brain extracts of mutations impairing only CK2α function ($CK2\alpha^{Tik}$) (Figure 3 H). We were able to restore CK2β levels by expression of wild type or kinase-dead CK2α demonstrating that CK2α abundance but not kinase activity controls CK2β protein levels (Figure 3 H). Similarly, presynaptic knock down of CK2β resulted in a reduction in CK2α levels (Figure 2K). These results indicate that a direct interaction between the two subunits is essential for CK2 complex stability *in vivo*. A recent biochemical study of the human CK2 complex identified two CK2α residues that are essential for the association with CK2β *in vitro*. Alanine substitutions of leucine 41 and phenylalanine 54 severely impair association with CK2β without affecting kinase activity (Raaf et al., 2011). These residues are perfectly conserved in Drosophila CK2α (corresponding to L39 and F52) enabling us to directly test potential *in vivo* requirements of these interaction sites (Figure 5 A).

We first tested whether alanine substitutions of these residues disrupted the CK2α-CK2β interaction in immunoprecipitation (IP) assays. We generated tagged constructs of wild type, single and double mutant proteins and performed IP-assays using Drosophila S2-cell extracts. Using GFP-tagged CK2α we were able to successfully precipitated HA-tagged CK2β. In contrast, the single point mutations (L39A and F52A) reduced and the double mutation (L39A*F52A) almost abolished the CK2α-CK2β interaction (Figure 5 B). As expected from our *in vivo* rescue experiments (Figure 3 H) the kinase-dead mutation of CK2α (K66M) did not impair this interaction (Figure 5 B). We then used phi31-mediated site-specific integration (attP40) (Bischof et al., 2007) to generate transgenic flies carrying both single and and the double mutation of CK2α. To address the consequences of these mutations on the CK2α-CK2β interaction we expressed the constructs ubiquitously in the background of the strong $CK2\alpha^{P2}$ mutation. The site-specific integration ensured identical expression CK2α levels in these animals (Figure 5 C). Interestingly, the two single mutations, that displayed a strong reduction in CK2β interaction in our S2-cell IP experiments (Figure 5 B) and that disrupted the interaction *in vitro* for human CK2 (Raaf et al., 2011), did not impair the interaction *in vivo* and completely restored CK2β levels (Figure 5 C). In contrast, the double mutant construct failed to rescue the loss of CK2β protein despite presence of equivalent CK2α protein levels (Figure 5C). We next tested the ability of these constructs to rescue the synapse retractions of CK2α mutants. Both single mutations completely rescued synapse stability but we observed only a partial rescue when using the double mutant construct. Similarly, ubiquitous expression of both single mutations but not of the double mutant construct restored viability of CK2 mutations (data not shown; or Supplemental table 2). Together our data indicate that L39 and F52 cooperatively mediate the interaction between CK2α and CK2β *in vivo* and this interaction is essential interaction for synapse stability and viability.

We then addressed the localization of CK2α and CK2β within motoneurons. As CK2α and CK2β antibodies did not work *in situ* we generated UAS-GFP-tagged versions of CK2α and CK2β. Expression in motoneurons resulted in efficient localization to synaptic bouton and inter-bouton regions within the presynaptic nerve terminal, partially overlapping with synaptic vesicle domains (Figure 5 E). Importantly, expression of GFP-CK2α was able to rescue synaptic

retractions and viability of *CK2α* mutations (data not shown) demonstrating that the observed localization represents functional CK2α distribution (Figure 5 E, second column).

CK2 controls synapse stability independently of PP2A

We next investigated whether CK2 might control synapse stability by regulating or interacting with other kinases or phosphatases identified in our screen. The prime candidate was PP2A as a CK2α can directly bind to and activate PP2A (Heriche et al., 1997). Furthermore, a residue essential for this CK2-PP2A interaction, E165, is mutated in $CK2\alpha^{Tik}$ (E165D). This allele carries an additional mutation, M161K (Figure 6 A). Previous analysis of PP2A function at the NMJ using ectopic expression of a dominant version of the catalytic active subunit Mts demonstrated a requirement of PP2A for normal synapse development but did not report alterations in synapse maintenance (Viquez et al., 2009). In our screen we identified the structural subunit PP2A-29B as essential for synapse stability. RNAi-mediated knock down using two dsRNA lines targeting independent sequences of PP2A-29B resulted in synaptic retractions at more than 40% of analyzed NMJs (Figure 6 C, G); Figure 1 B, G, H; Supplemental Figure 1 B, G; Supplemental Figure 2B; Supplemental table 2). Indeed, in animals expressing the dominant-negative catalytic subunit Mts we observed a significant increase in synaptic retractions in addition to the published synapse formation defects (Figure 6 D, E, G). Similarly, we detected synapse formation defects after knock down of PP2A-29B (Figure 6 F). Thus, PP2A controls both synapse formation and stability. To test whether the CK2-PP2A interaction is required for synapse maintenance we generated a series of mutations in this domain, mimicking the two Tik mutations, mutating the E165 to alanine or mutating all three essential residues E165R167K168 to alanine, thereby abolishing the CK2α-PP2A interaction (Heriche et al., 1997) (Figure 6 A). Surprisingly, the CK2α interaction with PP2A is not essential for synapse maintenance as all synaptic defects can be rescued with the triple mutant construct (ENRK165ANAA). In contrast, CK2αM161K failed to completely rescue the synaptic retraction phenotype (Figure 6 H) indicating that this amino acid exchange renders CK2α non-functional. Our data demonstrate that a direct interaction between CK2 and PP2A is not essential for synapse stability.

CK2 maintains the synaptic localization of essential synaptic proteins

Next we aimed to identify potential cellular targets that depend on CK2 function to maintain synapse stability. We hypothesized that the synaptic localization of CK2 targets causal for the synapse stability defects should already be altered at still stable synapses that do not yet show any signs of synaptic retractions. Thus we analyzed only presynaptic nerve terminals that displayed opposing pre- and postsynaptic markers throughout the entire presynaptic nerve terminal. We used the synaptic vesicle marker DvGlut as the presynaptic marker as DvGlut displays high sensitivity to synapse disassembly and can be used to define distal, middle and proximal regions of the presynaptic nerve terminal. We focused our analysis on cell adhesion and cytoskeletal proteins that have previously been implicated in the control of synapse stability. These molecules include the NCAM-homolog Fasciclin II (FasII), the L1-type CAM Neuroglian (Nrg) and the adaptor molecule Ankyrin2-L (Ank2-L) (Koch et al., 2008; Pielage et al., 2008; Schuster et al., 1996). Analysis of synaptic localization revealed a striking reduction of Nrg and Ank2-L but not of FasII levels in presynaptic nerve terminals of CK2α mutants. The reduction was most severe at distal boutons but was significant throughout the entire nerve terminal for Nrg and Ank2-L but not for FasII (Figure 7 A, B, D, E, G, H, J). At these terminals we did not observe any significant changes in DvGlut levels demonstrating that the NMJs are still stable. This reduction in Nrg and Ank2-L levels at distal boutons was evident at all NMJs within CK2α mutant animals regardless of the muscle group analyzed. This reduction represented a local alteration of protein distribution because we did not observe a reduction in total Nrg180 protein in larval brain extracts (Figure 7 K). Importantly, presynaptic expression of wild type but not of kinase-dead CK2α was sufficient to restore Nrg and Ank2-L levels in all synaptic boutons (Figure 7 G, H). As vertebrate L1 CAM is a direct target of CK2α (Wong et al., 1996) we next tested whether UAS-Gal4-mediated expression of Nrg in the background of CK2α mutations might be sufficient to restore presynaptic Nrg levels and synapse stability. While expression of Nrg restored presynaptic Nrg levels in (Figure 7 C, G) it failed to rescue the impairment in synapse stability (Figure 7 L). Similarly, this ectopic increase of presynaptic Nrg was not sufficient to restore Ank2-L levels in these animals (Figure 7 F, H). Thus ectopic Nrg expression can overcome the impairment of Nrg distribution in CK2 mutants but is not sufficient to restore synapse stability or presynaptic Ank2-L levels.

CK2 controls synaptic stability via Ank2-L

If phosphorylation of Ank2-L is indeed required to control synapse stability, overexpression of presynaptic CK2α might be able to alleviate synaptic retractions caused by partial loss of Ank2. The hypomorphic ank2-L allele ank2^{f02001} (abbreviated *ank2*^{P2}) is a piggybac insertion in the ank2-L-isoform specific exon causing a trunctation of the protein after amino acid 2518 (www.flybase.org; Ank2-L wild type protein consists of 4083 amino acids). This mutation causes a significant increase in synaptic retractions compared to wild type (Figure 8 B, E, F; (Pielage et al., 2008)). Indeed, presynaptic expression of wild type CK2α in *ank2*^{P2} mutant animals significantly alleviated the synaptic retraction phenotype (Figure 8E, F). In contrast, expression of CK2α^{KD} further enhanced synaptic retraction phenotypes in *ank2*^{P2} mutants (Figure 8 C-F). This is most likely due to competition with residual CK2α protein in this genetic background. Our data demonstrates that CK2 activity can modulate synaptic retractions caused by a partial loss of Ank2-L. To further validate the these findings we performed genetic interaction experiments. While we did not observe a significant increase in synaptic retractions in trans-heterozygous *CK2α*$^{P1/+}$; *ank2-L*$^{P2/+}$ animals, removal of one copy of *CK2α* in *ank2*^{P2} mutant animals or removal of one copy of *ank2* in *CK2α*^{P1} mutant animals significantly increased the frequency of synaptic retractions (Figure 8G, H). Together these experiments identify Ank2-L as a potential target of CK2 *in vivo*. Therefore, we next analyzed the Ank2-L open reading frame for potential CK2 phosphorylation sites (serine or threonine residue in an acidic environment e.g. S/TXXE/D) using the web tool KinasePhos ((Huang et al., 2005); http://KinasePhos.mbc.nctu.edu.tw). The KinasePhos program identified up to 135 serine residues and 48 threonine residues as potential CK2 phosphorylation sites within the Ank2-L protein sequence (cut off set to > -6). We previously identified a domain in Ank2-L that can directly bind to microtubules and thus might contribute to synapse stability (Ank2^{MT} = aa 1682-2089) (Pielage et al., 2008). We purified this domain as a His-tagged fusion protein to test whether CK2 can phosphorylate Ank2-L. The Ank2^{MT} domain contains 15 predicted serine and 9 predictd threonine phosphorylation sites. Using γ-ATP as a substrate for purified human CK2 (New England Biolabs) we observed efficient phosphorylation of Ank^{MT} *in vitro*. This phosphorylation can efficiently be inhibited by the addition of 30 μM of the CK2-specific inhibitor TBB (4,5,6,7-Tetrabromo-2-azabenzimidazole) (Figure 8 I) (Sarno et al., 2001).

Finally, we analyzed the reactions by mass spectroscopy to identify the actual CK2 phosphorylation sites. We identified three residues that are efficiently phosphorylated by CK2 and that are sensitive to the presence of the specific inhibitor TBB. These sites are Ank2-L S1686, S1780 and S1883. All sites represent bona fide CK2 phosphorylation sites with a SXXE consensus sequence (Figure 8 J). Ank2-L thus represents a likely target for CK2 to control aspects of synapse morphology and stability.

Discussion

Local regulation of synaptic connectivity enables the adjustment of neuronal circuits in response to sensory or motor activity including learning and memory paradigms. Here we systematically screened the kinome and phosphatome of Drosophila for regulators of synapse stability. We identified 11 kinases and phosphatases that are essential for the maintenance of synaptic connections. These kinases and phosphatases can be grouped into three main categories identifying phospholipid signaling, metabolic signaling and modulation of the presynaptic cytoskeleton as key signaling nodes controlling synaptic connectivity. The analysis of a major regulatory molecule identified in the screen, Casein kinase 2 provides novel insights into the mechanisms controlling the regulation of synapse stability. CK2 is a constitutive active kinase and we identify regulation of CK2α-CK2β subunit interaction and expression levels as essential mechanisms controlling presynaptic kinase activity. We then identified the adaptor molecule Ankyrin2 as an essential CK2 target necessary for the control synapse stability. Ank2 provides a regulatable link between synaptic cell adhesion molecules and the actin and microtubule cytoskeleton. We propose a model where constitutive control of protein-protein interactions essential for synapse maintenance provides an attractive mean for the precise control of the lifetime of synaptic connections. A local modulation of the constitutive, phosphorylation – dependent equilibrium would enable precise temporal and spatial control of synaptic connectivity without impairing overall neuronal architecture.

Identification of signaling pathways controlling synapse stability

Reversible phosphorylation of proteins represents a major mechanism to regulate many aspects of synaptic development and function, including processes underlying learning and memory (Mayford, 2007; Siddiqui and Craig, 2011; Soderling and Derkach, 2000; Winder and Sweatt, 2001; Wu et al., 2010). Although it is generally believed that a memory trace is

established and maintained by alterations in synaptic connections (Kandel, 2001), to date we know only few kinases and phosphatases involved in the molecular mechanisms ensuring synapse stabilization and maintenance (Caroni et al., 2012). In our screen we identified 7 kinases (CK2, CKIα, Mnb, PI4KIIIα, InR, MAST, Cdc2) and 4 phosphatases (PP2A-29B, CG9784, PP4-19C, Mipp2) that are essential for synapse stabilization. To our knowledge, none of these molecules were previously directly implicated in synapse stability. Based on their main function the identified kinases and phosphatases can be grouped into the following signaling pathways: Regulation of phospholipids (PI4KIIIa, Mipp2, CG9784), metabolic signaling (InR, Mnb), and regulation of the cytoskeleton (CK2, MAST, Cdc2, PP2A, PP4).

Inositol kinase PI4KIIIα and the two inositol phosphatases CG9784 and Mipp2 regulate the phosphorylation status of phosphoinositides, essential lipids that are present in the cytoplasmic leaflet of the plasma membrane. Regulation of phosphoinositides plays crucial roles in the control and assembly of signaling complexes, cell adhesion, cytoskeletal dynamics and as such is essential for nervous system development (Di Paolo & De Camilli, 2006; Lo Vasco 2012; Vicinanza, D'Angelo, Di Campli, & De Matteis, 2008). In particular, PI4KIIIα has been implicated to negatively regulate actin dependent growth in a WSP-dependent manner by increasing the presynaptic PI(4,5)P_2 levels (Khuong et al., 2010). Indeed, in addition to defects in synapse stability we observed an increase in NMJ growth in our PI4KIIIα knock downs implicating regulation of the presynaptic actin cytoskeleton via modification of phospholipids in the control of synapse maintenance.

Insulin Receptor (InR) and a serine/threonine kinase Minibrain (Mnb) represent important regulators of metabolic signaling (Hong et al., 2012). The insulin receptor pathway plays an important role in regulation of synaptic plasticity, neuronal survival, axon guidance, neurite outgrowth, spine density, synapse activity, spatial memory, and cognitive function (Choi et al., 2005; Govind et al., 2001; Huang et al., 2010; Huang et al., 2003; Zhao et al., 1999). Indeed, modulating InR levels through expression of wild type or dominant-negative constructs directly correlated with up- or downregulation of the number of synaptic boutons at the Drosophila NMJ, however these functions were attributed to synaptogenic and not stabilizing functions (Martin-Pena et al., 2006). The mammalian ortholog of Mnb, the Dual specificity tyrosine-phosphorylation-regulated kinase 1a (Dyrk1a) is, like Mnb, essential forneuroblast proliferation and brain development in mice (Fotaki et al., 2002). Importantly, human patients

with truncated versions of Dyrk1a have mental retardation and microcephaly (Moller et al., 2008; van Bon et al., 2011). Thus, readout of metabolic states and in particular insulin receptor signaling might directly impinge on the control of synaptic connectivity.

The precise regulation of the actin and microtubule cytoskeleton is essential for all processes depending on changes of cell morphology. Thus, it is not surprising that we identified a number of kinases and phosphatases that have been directly implied as regulators of actin and microtubule dynamics. Of special interest are regulators of the microtubule cytoskeleton as alterations in the microtubule cytoskeleton appear to be one of the earliest steps in regulated and disease associated synapse elimination (Eaton et al., 2002; Pielage et al., 2008; Pielage et al., 2005). Three serine/threonine protein kinases MAST, Cdc2, and CK2 and two serine/threonine protein phosphatases PP2A and PP4 from the RNAi screen are known to bind directly to microtubules and to various microtubule-associated proteins (MAPs) (Besson et al., 2004; Fourest-Lieuvin et al., 2006; Han et al., 2009; Helps et al., 1998; Lemos et al., 2000; Lim et al., 2004; Sontag et al., 1995; Sontag and Sontag, 2006; Toyo-oka et al., 2008; Wang et al., 2012). The detailed analysis of the signaling pathways associated with the identified regulatory molecules and identification of their relevant presynaptic targets will provide new insights into the function of the microtubule cytoskeleton in synapse assembly and disassembly.

CK2 controls synapse stability via Ankyrin2

We focused our study on the role of the serine/threonine kinase CK2 in synapse stability. We demonstrated that both the catalytic subunit CK2α and the regulatory subunit CK2β are essential for synapse maintenance at the Drosophila NMJ. CK2 has the potential to directly bind to microtubules, microtubule-associated proteins (MAPs), and other neuronal cytoskeletal proteins including myosin heavy chain, Tau, β-spectrin, and L1/Neuroglian (Bignone et al., 2007; Diaz-Nido et al., 1988; Greenwood et al., 1994; Matsumura et al., 1983; Serrano et al., 1987; Wong et al., 1996). CK2 thus represents an exciting candidate to sustain synapse stability by controlling protein-protein interactions within the presynaptic cytoskeletal network.

Here, we identified Ankyrin2, an adaptor molecule linking synaptic cell adhesion molecules to the cytoskeleton, as a CK2 substrate for maintaining synapse stability. Ank2 has previously been implicated in synapse stability (Pielage et al., 2008). However, to date, nothing is known regarding potential post-translational mechanisms that could regulate Ank2 binding to

synaptic constituents and hence physically stabilize or disrupt the cytoskeletal network. We first demonstrated that presynaptic localization of Ank2 is controlled by presynaptic CK2 activity. Most likely, CK2 phosphorylation of Ank2 ensures its proper synaptic localization by enhancing its association with presynaptic constituents, such as microtubules and/or the cell adhesion molecule Nrg, which is also essential for synapse stability (Enneking et al 2013, in press). Indeed, we demonstrated that Nrg levels were diminished in CK2 mutants. However, expression of Nrg in the CK2 mutant background was not sufficient to restore Ank2 localization or the synaptic retraction phenotype thus indicating that Nrg levels were indirectly perturbed through the impairment of Ank2 localization (in agreement with Pielage et al., 2008; Enneking et al., 2013). We showed that CK2 phosphorylates a domain of Ank2-L *in vitro*, which directly binds to microtubules and therefore might contribute to synapse stability *in vivo*. In support of this model, we demonstrated that enhancing CK2 activity in *ank2* mutants significantly alleviated the retraction phenotype, whereas decreasing CK2 activity enhanced synapse disassembly. Possibly, increased CK2 phosphorylation of the truncated Ank2 protein was able to enhance its binding to microtubules and/or other key synaptic proteins and thus increase stability of the presynaptic network. By demonstrating a direct genetic interaction between CK2α and Ank2 we further verified their collaboration in the regulation of synaptic stabilization. Collectively, our data strongly imply Ank2 as a key target of CK2 to regulate synapse stabilization.

Most protein kinases are turned on only in response to specific stimuli. CK2, on the other hand, is a constitutively active kinase and hence it might control synapse stability via persistent enzymatic molecular mechanisms. Thus far, a similar function has only been proposed for one other constitutively active kinase, PKMζ, which has been thought to control long-term memory maintenance through continuous stabilization of neurotransmitter receptors at synaptic sites. However, this data dependent on the application of a specific inhibitor of PKMζ, and recent studies using knock-out mutations demonstrated that PKMζ is not essential for long term memory (Lee et al., 2013; Volk et al., 2013). Our data indicate that CK2 could maintain synapse stability through continuously phosphorylating Ank2 and/or other cytoskeletal proteins, hence linking cytoskeletal signaling to the maintenance of the long-term morphology of synaptic connections. Considering that vertebrate CK2 is especially enriched in brain regions directly implicated in learning and memory (Blanquet, 2000; Kimura and Matsuki, 2008), CK2

represents an exciting candidate for playing an essential role in mechanisms underlying learning and memory. Intriguingly, another hit in our RNAi screen represents an additional, constitutively active kinase, CKIα. CKIα belongs to the same kinase family as CK2 and also has a preference to acidic substrates (Morrison et al., 2000). Together, this implicates that maintenance of posttranslational modifications via constitutively active kinases might provide means to precisely control synapse stabilization. An attractive hypothesis is that this constitutive equilibrium of phospho-modifications of synapse stability molecules enables the precise temporal and spatial control of synapse stability by modulating either kinase activity or local activation of the antagonistic phosphatase.

Regulation of CK2 activity

We provide evidence for two mechanisms essential for the regulation of presynaptic activity of CK2. First, we identified the CK2α-CK2β subunit interaction as essential for the control of kinase function *in vivo*. An important question is whether the formation of the CK2 α_2/β_2–holoenzyme represents a reversible process within the cell and how this process might be regulated. There have been a number of conflicting studies as to whether the isolated CK2α and CK2β subunits can exist and function in cells *in vivo* (Canton et al., 2001; Filhol et al., 2003; Guerra et al., 1999; Luscher and Litchfield, 1994; Stigare et al., 1993). We demonstrated that in Drosophila, proper CK2α-CK2β binding is absolutely required for the stability of CK2 holoenzyme complex *in vivo*. Loss of CK2α caused a dramatic reduction in CK2β protein levels. Likewise, knock down of CK2β caused diminished CK2α protein levels. Moreover, expressing a CK2α construct that is not able to bind efficiently to CK2β could rescue neither the lethality nor the synaptic retraction phenotype of CK2α mutants. This is consistent with a previous study demonstrating that proper CK2α-CK2β interaction is absolutely essential for fly viability, as mutations in CK2β abolishing the holoenzyme formation could not rescue lethality of CK2β loss of function mutant (Jauch et al., 2002). In our study, we provided evidence that synapse stability could be locally controlled via regulation of CK2α-CK2β association. Therefore, local activation of yet unidentified molecules that would selectively disrupt CK2α-CK2β association could be an important mechanism for down-regulating CK2 levels in a confined region, hence causing local synapse disassembly. In addition to synaptic retractions, we also observed dramatic changes in

synaptic bouton number and morphology in CK2α mutant animals. In contrast, animals expressing high levels of CK2α showed the exact opposite phenotype. This further indicates that CK2 can directly regulate the organization of the presynaptic cytoskeleton. Indeed, we previously identified an almost identical phenotype like the CK2 overexpression phenotype in animals expressing only a c-terminal domain of Ank2 (Pielage et al., 2008). This fragment included both the Ank2 microtubule domain and the c-terminal domain but not the membrane association domains thereby potentially lacking any regulation of local activity. Thus, regulation of CK2α expression levels represents an alternative mechanism to control its presynaptic activity.

CK2 and neurodegenerative disease

Protein kinase CK2 is highly conserved and is found in all eukaryotic cells described to date (Litchfield, 2003). Our work provides evidence that CK2 might play a major role in assembly of cytoskeleton-related multiprotein complexes and synchronizing signaling pathways underlying synaptic plasticity events. Interestingly, CK2 activity and microtubule-associated protein phosphorylation have been implicated in the development of a number of neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington Diseases (Perez et al., 2011). Indeed, prior studies demonstrated decreased levels of CK2 and aberrations in the phosphorylation of structural proteins the cortex of schizophrenic and Alzheimer's disease patients (Aksenova et al., 1991; Iimoto et al., 1990). CK2 activity was also shown to decline in the hippocampal CA1 region and striatum after brief ischemia (Hu and Wieloch, 1993). Moreover, activators of CK2 were demonstrated to protect neural cells against ischemic damage (Gilad and Gilad, 1991). It will thus be of high interest to investigate the role of CK2 in synapse stability and plasticity in vertebrate model systems using conditional mutations. Our study presents the first indication that CK2 might represent a key molecule controlling the life-time of synaptic connections.

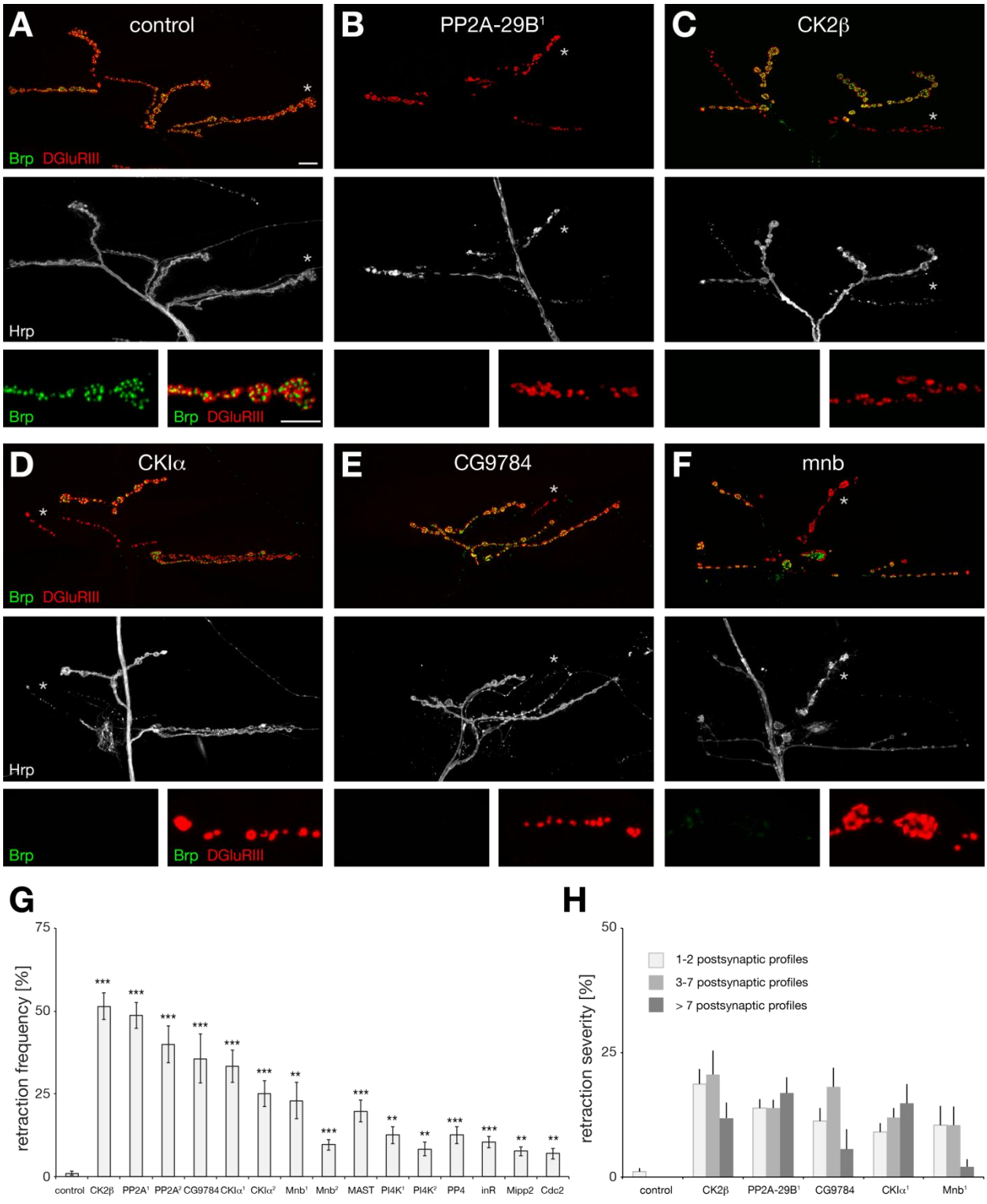
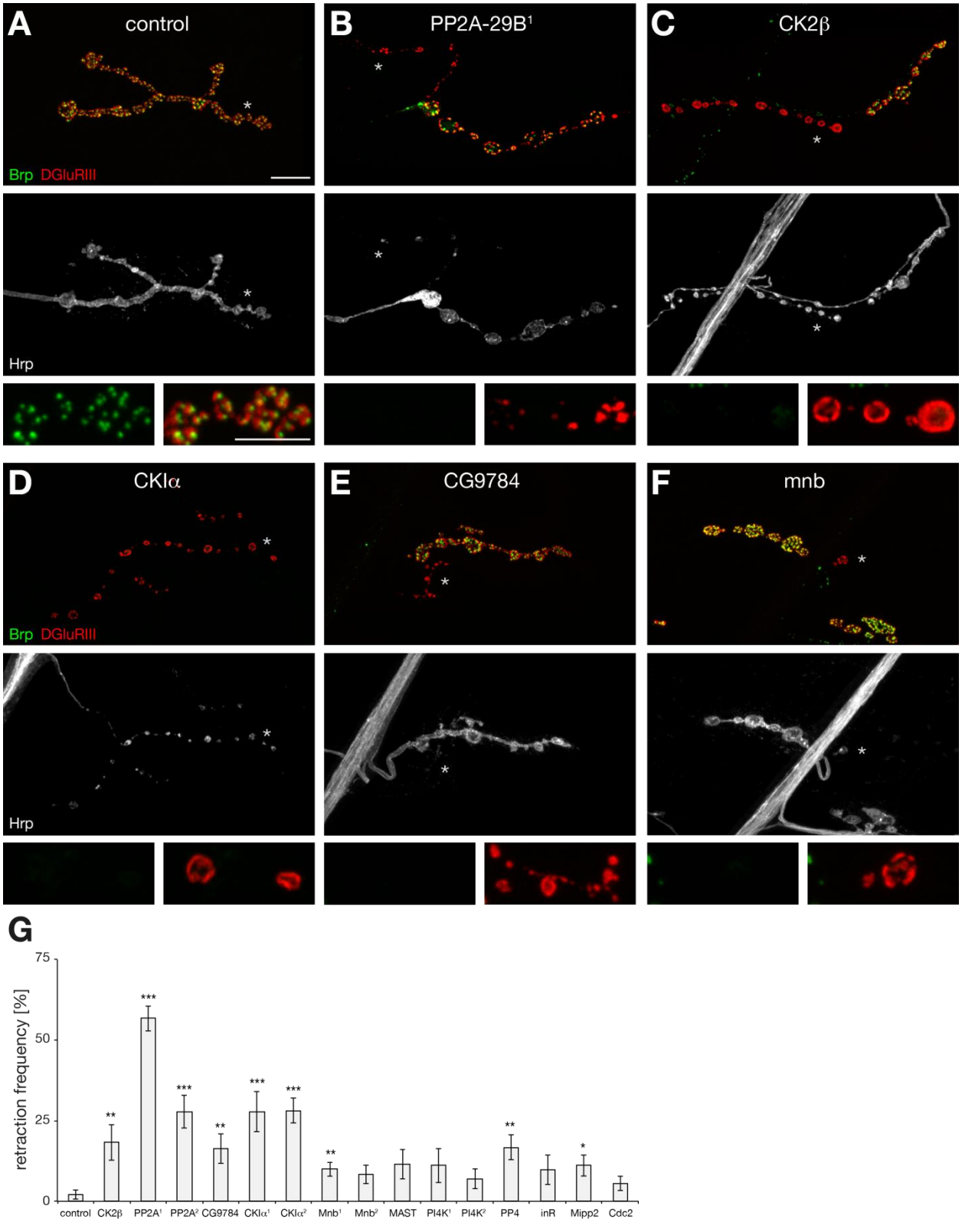


Figure 1 – RNAi screen identifies kinases and phosphatases essential for synapse stability (muscles 1/9, 2/10 marked by Brp, DGluRIII, and Hrp)

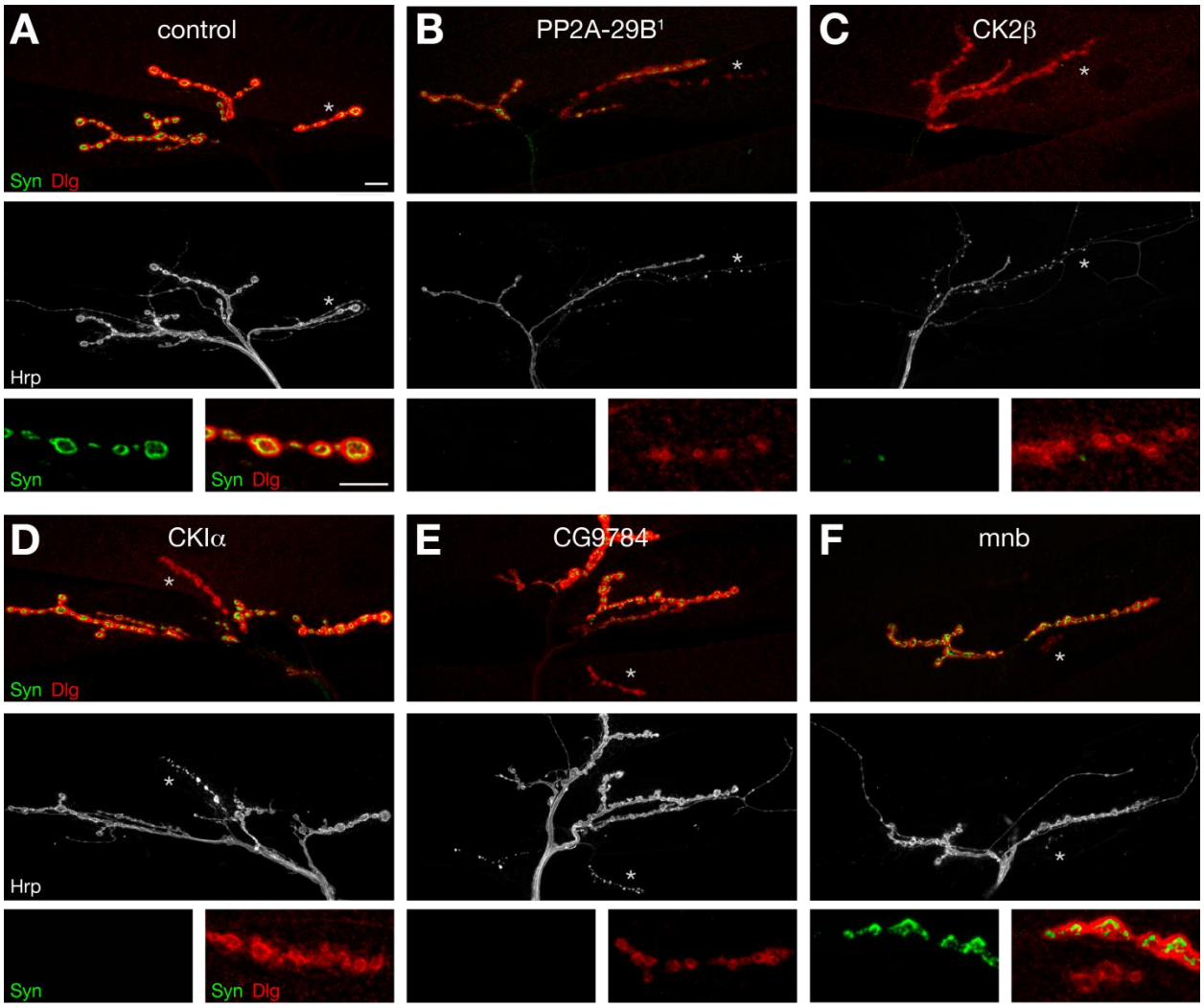
(A-F) NMJs on muscles 1/9 or 2/10 stained for the presynaptic active zone marker Bruchpilot (Brp, green), postsynaptic glutamate receptors (DGluRIII, red), and the presynaptic motorneuron membrane

marker Hrp (white). Scale bars: overviews 10 μm, insets 5 μm. **(A)** A stable wild type NMJ on muscle 1/9 is indicated by a precise apposition of pre- and postsynaptic markers. **(B-F)** Knock down of presynaptic PP2A-29B, CK2β, CKIα, CG9784, or Mnb using RNAi caused a retraction phenotype on muscles 1/9 or 2/10. Postsynaptic glutamate receptors (DGluRIII) lacked apposing presynaptic active zones (Brp) (insets). The presynaptic membrane (Hrp) was no longer continuous and only remnants of the membrane were visible opposite postsynaptic glutamate receptors at the sites of retractions. One example (B) shows a complete elimination of an entire NMJ on muscle 2/10. **(G)** Quantification of synaptic retraction frequency on muscles 1/9, 2/10 in different RNAi lines. Significant differences between compared groups are noted by asterisks (**≤0.01, ***≤0.001, n=9-15 animals). Knock down of 7 kinases (CK2β, CKIα, Mnb, MAST, PI4K, inR, and Cdc2) and 4 phosphatases (PP2A, CG9784, PP4, and Mipp2) resulted in a significant increase in synaptic retractions. Two independent RNAi lines were used for 4 candidates (PP2A, CKIα, Mnb, and PI4K). **(H)** Quantification of synaptic retraction severity on muscles 1/9, 2/10.



Supplemental Figure 1 - RNAi screen identifies kinases and phosphatases essential for synapse stability (muscle 4 marked by Brp, DGluRIII, and Hrp)

(A-F) NMJs on muscle 4 stained for the presynaptic active zone marker Brp (green), postsynaptic glutamate receptors DGluRIII (red), and the presynaptic motorneuron membrane marker Hrp (white). Scale bars: overviews 10 μm, insets 5 μm. **(A)** A stable wild type NMJ on muscle 4 is indicated by a precise apposition of pre- and postsynaptic markers. **(B-F)** Knock down of presynaptic PP2A-29B, CK2β, CKIα, CG9784, or Mnb using RNAi caused a retraction phenotype on muscle 4 indicated by the fragmentation of the presynaptic membrane and the loss of presynaptic Brp despite the presence of postsynaptic glutamate receptors (insets). The severity of retractions in examples shown here ranges from one bouton retraction (F) to elimination of entire branches (B-D). One example (D) shows a complete elimination of an entire NMJ on muscle 4. **(G)** Quantification of synaptic retraction frequency on muscle 4 in different RNAi lines. Significant differences between compared groups are noted by asterisks (* ≤0.05, **≤0.01, ***≤0.001, n=9-15 animals). Knock down of 3 kinases (CK2β, CKIα, Mnb) and 4 phosphatases (PP2A, CG9784, PP4, and Mipp2) resulted in a significant increase in synaptic retractions on muscle 4 (P≤0.05).



Supplemental Figure 2 - RNAi screen identifies kinases and phosphatases essential for synapse stability (muscles 1/9, 2/10 marked by Syn, Dlg, and Hrp)

(A-F) NMJs on muscle 1/9 stained for the presynaptic vesicle marker Synapsin (Syn, green), postsynaptic density marker Discs-large (Dlg, red), and the presynaptic motorneuron membrane marker Hrp (white). Scale bars: overviews 10 μm, insets 5 μm. **(A)** A stable wild type NMJ on muscle 1/9 is indicated by a precise apposition of pre- and postsynaptic markers. **(B-F)** Knock down of presynaptic PP2A-29B, CK2β, CKIα, CG9784, or Mnb using RNAi caused a retraction phenotype on muscle 1/9. Postsynaptic Dlg lacked apposing presynaptic Syn (insets). The presynaptic membrane (Hrp) was no longer continuous and only remnants of the membrane were visible opposite postsynaptic Dlg at sites of retractions. One example (C) shows a complete elimination of an entire NMJ on muscle 1/9.

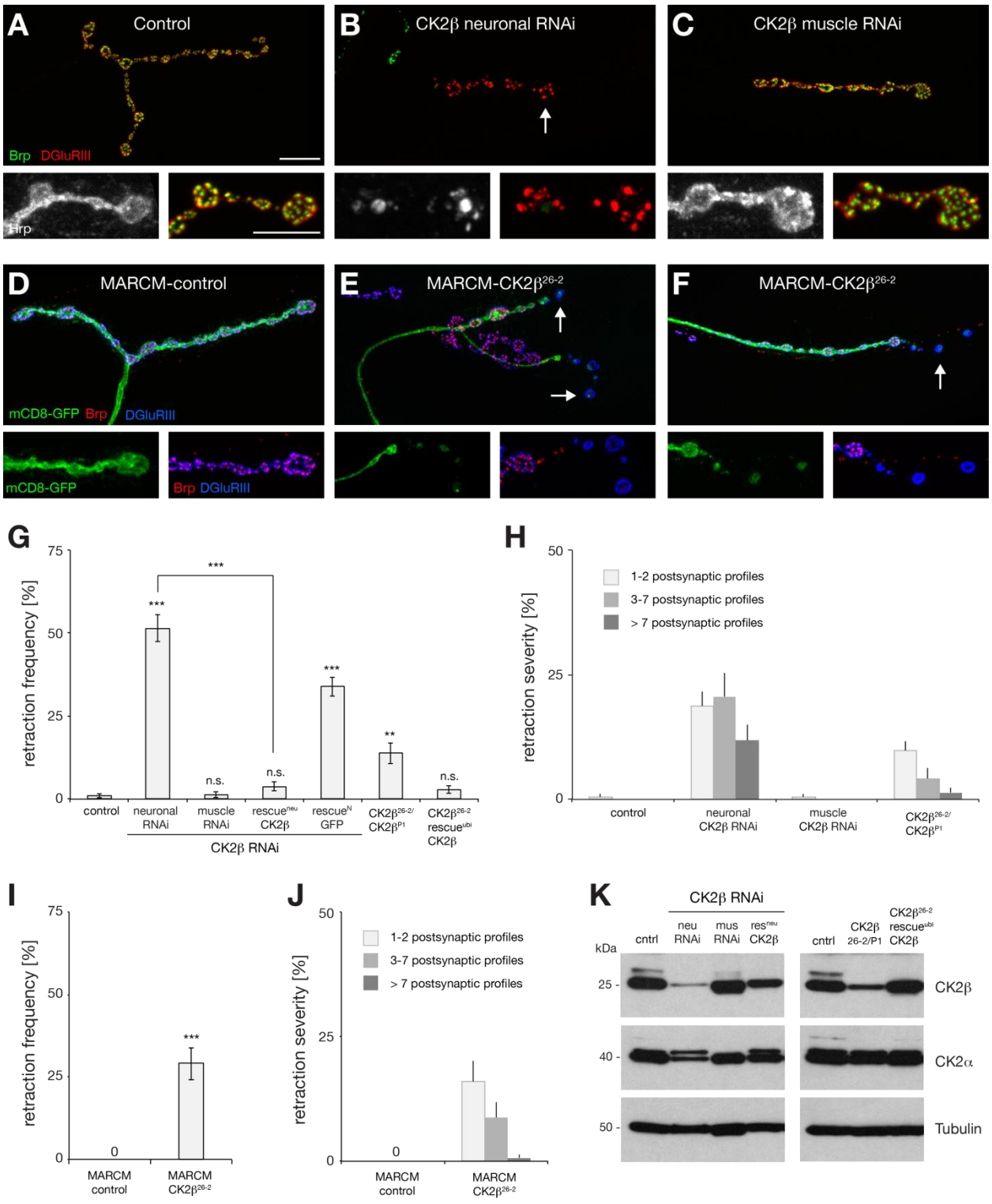


Figure 2 – Presynaptic CK2β is essential for synapse stability

(A-C) NMJs on muscle 4 stained for the presynaptic active zone marker Brp (green), postsynaptic glutamate receptors DGluRIII (red), and the presynaptic motorneuron membrane marker Hrp (white). Scale bars: overviews 10 µm, insets 5 µm. **(A)** A stable wild type NMJ on muscle 4 is indicated by a

precise apposition of pre- and postsynaptic markers. **(B)** Loss of presynaptic CK2β resulted in a severe synaptic retraction phenotype indicated by the loss of presynaptic Brp and the fragmentation of the presynaptic membrane (Hrp). **(C)** Knock down of muscle CK2β did not impair synapse stability. **(D-F)** Motorneuron control and CK2β-null MARCM clones are marked by the expression of membrane-bound GFP (mCD8-GFP, green). Presynaptic active zones are marked by Brp (red) and postsynaptic glutamate receptors are marked by DGluRIII (blue). Scale bars: overviews 10 μm, insets 5 μm. **(D)** A control MARCM clone showing a perfect apposition of pre- and postsynaptic markers indicating a stable NMJ. **(E-F)** A $CK2\beta^{26\text{-}2}$ MARCM clone showing retractions indicated by the absence of presynaptic active zones (Brp) and fragmented remnants of the membrane-bound GFP marker opposed by fused glutamate receptors (arrows). **(G)** Quantification of retraction frequency on muscles 1/9, 2/10. Significant differences between compared groups are noted by asterisks (**≤0.01, ***≤0.001, n=9-13 animals). Neuronal but not muscle-specific knock down of CK2β resulted in a significant increase in synaptic retractions compared to control animals. Presynaptic co-expression of CK2β but not mCD8-GFP restored synaptic stability in CK2β RNAi animals. $CK2\beta^{P1/26\text{-}2}$ mutant animals showed a significant increase in synaptic retractions. Animals ubiquitously expressing wild type CK2β in $CK2\beta^{26\text{-}2}$ background showed no signs of synaptic instability. **(H)** Quantification of retraction severity on muscles 1/9, 2/10. **(I)** Quantification of mCD8-marked NMJs that showed retractions (P≤0.001, n=17-33 animals). We never observed synaptic retractions in control MARCM clones. **(J)** Quantification of retraction severity in mCD8-marked NMJs. **(K)** Western Blot analysis of larval brain extracts of the same genotypes as in (G) probed with antibodies against CK2β, CK2α, and Tubulin. Neuronal but not muscle-specific knock down of CK2β led to an efficient reduction of CK2β and a modest reduction of CK2α protein levels. CK2β and CK2α levels were rescued by neuronal co-expression of wild-type CK2β. Brain extracts of $CK2\beta^{P1/26\text{-}2}$ mutants showed modestly reduced CK2β but normal CK2α protein levels. Animals ubiquitously expressing wild-type CK2β in $CK2\beta^{26\text{-}2}$ background showed normal CK2α and CK2β protein levels.

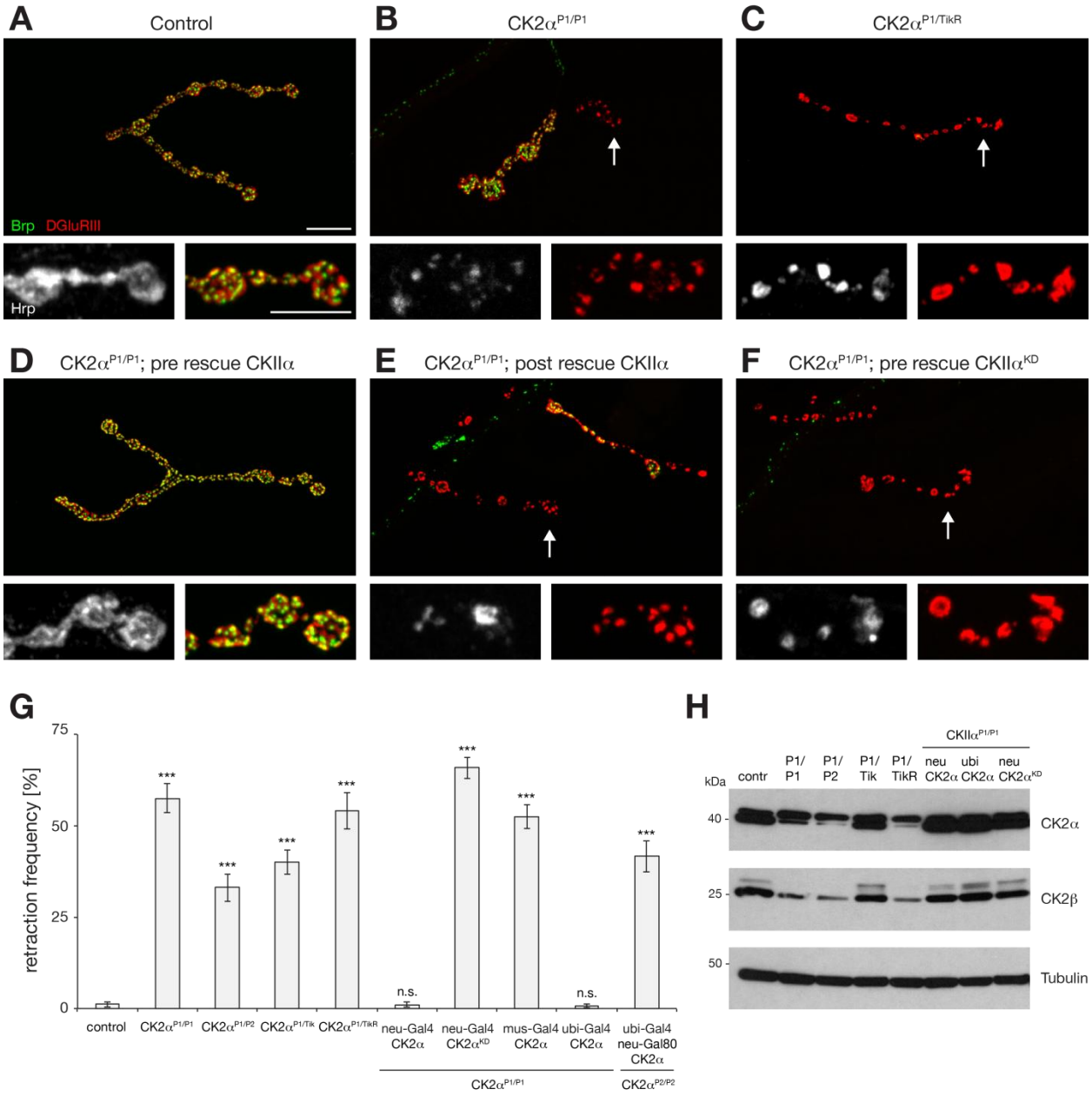
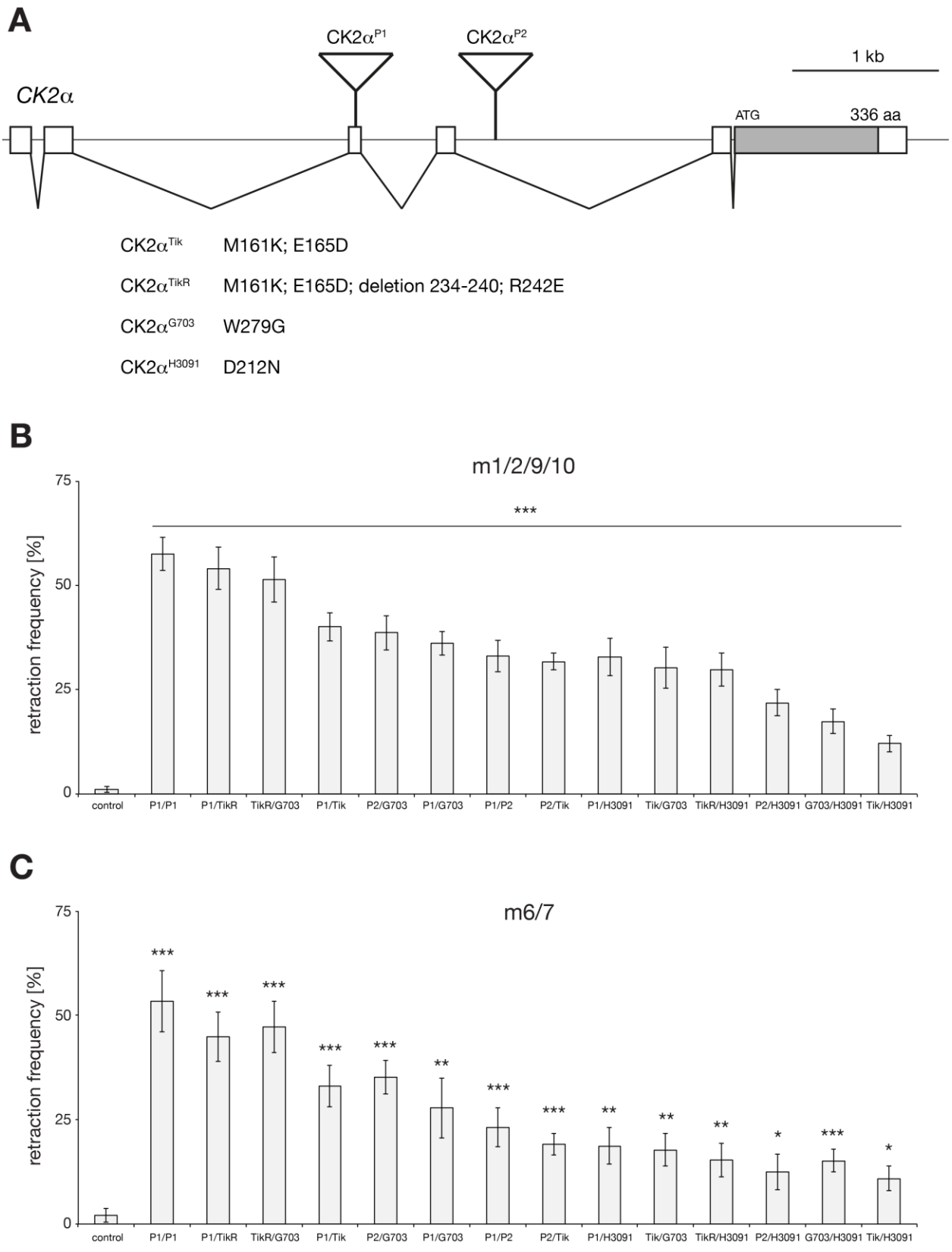


Figure 3 – Presynaptic CK2α is essential for synapse stability

(A-F) NMJs on muscle 4 stained for the presynaptic active zone marker Brp (green), postsynaptic glutamate receptors DGluRIII (red), and the presynaptic motorneuron membrane marker Hrp (white). Scale bars: overviews 10 µm, insets 5 µm. **(A)** A stable wild type NMJ on muscle 4 is indicated by a precise apposition of pre- and postsynaptic markers. **(B-C)** CK2α mutant animals showed synaptic instability phenotypes. CK2α$^{P1/P1}$ (B) and CK2α$^{P1/TikR}$ (C) are depicted as representative examples. Postsynaptic glutamate receptors (DGluRIII) were not apposed with presynaptic active zones (Brp) and the presynaptic membrane (Hrp) was fragmented. A less severe retraction affecting one branch (B) and a more severe elimination affecting an entire NMJ (C) are shown. **(D-E)** Neuronal but not muscle-specific expression of wild-type CK2α efficiently rescued the synaptic retraction phenotype in CK2α mutants.

The site of synaptic retraction (E) is indicated by an arrow. **(F)** Neuronal expression of kinase-dead CK2α was not sufficient to rescue synaptic instability as indicated by the loss of presynaptic Brp and the fragmentation of the presynaptic membrane (Hrp). A complete elimination of an entire NMJ is shown. **(G)** Quantification of retraction frequency on muscles 1/9, 2/10. Significant differences between compared groups are noted by asterisks (***≤0.001, n=9-22 animals). $CK2\alpha^{P1/P1}$, $CK2\alpha^{P1/P2}$, $CK2\alpha^{P1/Tik}$, and $CK2\alpha^{P1/TikR}$ mutant animals showed high frequencies of synaptic retractions compared to control animals. The retraction frequency was significantly rescued by presynaptic or ubiquitous, but not postsynaptic, expression of wild type CK2α. Presynaptic expression of kinase-dead CK2α was not able to rescue the phenotype. When we ubiquitously expressed wild-type CK2α but at the same time prevented CK2α neuronal expression in $CK2\alpha^{P2/P2}$ null mutants, the retraction frequency remained significantly high compared to wild type. **(H)** Western Blot analysis of larval brain extracts probed with antibodies against CK2α, CK2β, and Tubulin. A significant reduction of CK2α and CK2β protein levels was observed in $CK2\alpha^{P1/P1}$, $CK2\alpha^{P1/P2}$ and $CK2\alpha^{P1/TikR}$, but not in $CK2\alpha^{P1/Tik}$, mutant animals. CK2α and CK2β levels were restored by neuronal expression of wild type or kinase-dead CK2α. Ubiquitous expression of wild type CK2α was also sufficient to restore CK2α and CK2β levels.



Supplemental Figure 3 – Retraction frequencies of various CK2α mutant allelic combinations

(A) Schematic of the CK2α gene locus. Introns and exons are denoted in white and grey respectively. The position of the P-element insertion mutations are marked by triangles ($CK2\alpha^{P1}$ and $CK2\alpha^{P2}$). The amino acid substitutions and/or deletions are denoted for various CK2α mutant alleles. **(B and C)** Quantification of retraction frequency on muscles 1/9, 2/10 (B) and muscle 6/7 (C) of various CK2α mutant allelic combinations. Significant differences between compared groups are noted by asterisks (* ≤0.05, **≤0.01, ***≤0.001, n=9-37 animals). We observed a significant increase in the frequency of synaptic retractions for all listed allelic combinations compared to wild type animals. A synaptic retraction phenotype was slightly weaker but still highly significant on dorsal muscle 6/7 (C) in comparison to ventral muscles 1/9, 2/10 (B). The relative phenotypic strength remained identical for both muscle groups.

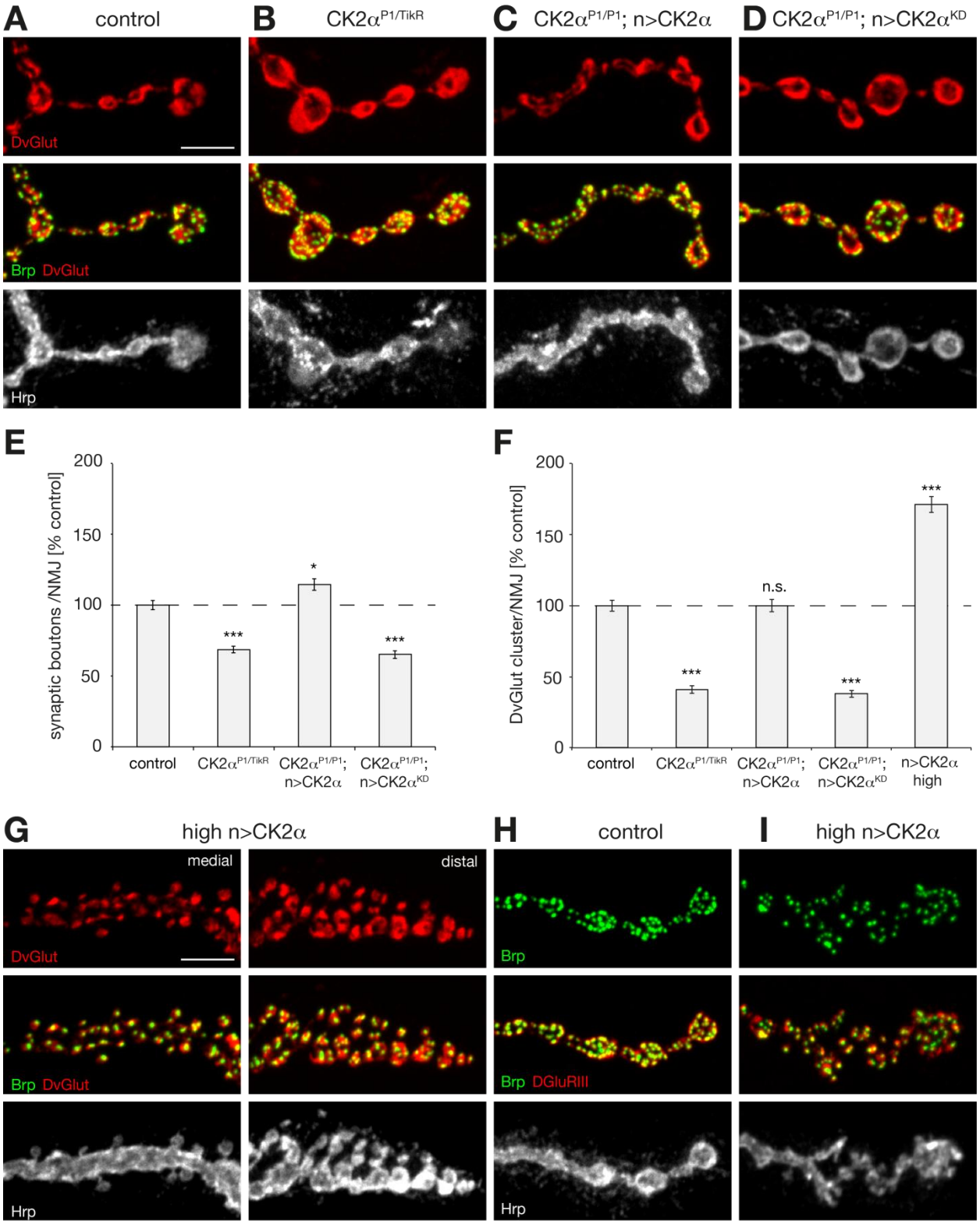


Figure 4 – Presynaptic CK2α controls organization of synaptic domains

(A-D, G) NMJs on muscle 4 stained for the presynaptic vesicle domain marker DvGlut (red), presynaptic active zone marker Brp (green), and the presynaptic motorneuron membrane marker Hrp (white). **(H)** NMJs on muscle 4 stained for the presynaptic active zone marker Brp (green), postsynaptic glutamate receptors (DGluRIII, red), and the presynaptic motorneuron membrane marker Hrp (white). Scale bar: 5 μm. **(A)** In wild type, every synaptic bouton is usually subdivided into two or more synaptic vesicle clusters marked by DvGlut. **(B)** In CK2α mutant animals synaptic vesicle domains were fused and not organized into separate clusters, and boutons appeared well-rounded. $CK2\alpha^{P1/TikR}$ is depicted here as a representative example. **(C-D)** The fused synaptic cluster phenotype was rescued by expression of wild type CK2α (C) but not kinase-dead CK2α (D) in CK2α mutant animals. **(E)** Quantification of synaptic bouton number on muscle 4. Values are normalized to wild type control. Significant differences between compared groups are noted by asterisks (* ≤0.05, ***≤0.001). CK2α mutant animals showed a significant 30% decrease in synaptic bouton number compared to wild type. Expressing wild type CK2α but not kinase-dead CK2α rescued the synaptic bouton number phenotype. **(F)** Quantification of DvGlut cluster number on muscle 4. Values are normalized to wild type control. Significant differences between compared groups were noted by asterisks (***≤0.001, n=48 NMJs). CK2α mutants showed a significant more than 60% reduction in the number of individual DvGlut-positive synaptic vesicle clusters compared to wild type. Expression of wild-type but not kinase-dead CK2α rescued the number of synaptic vesicle clusters. Overexpression of CK2α in wild type background caused a significant 1.7-fold increase in the number of synaptic vesicle domains compared to controls. **(G-H)** In animals expressing high levels of CK2α synapses were no longer organized into defined boutons and looked dispersed. NMJs became subdivided into smaller individual domains compared to wild type.

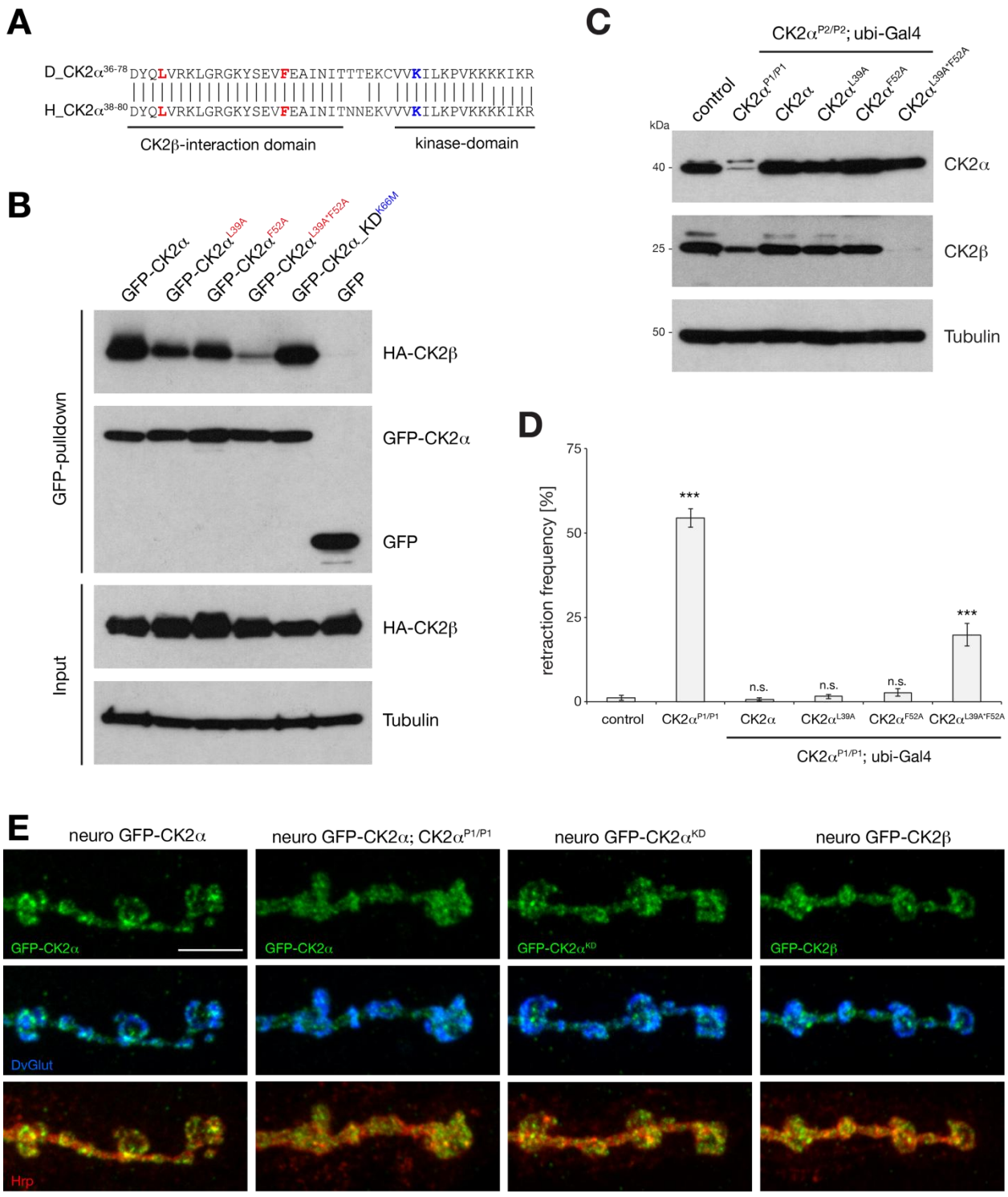


Figure 5 – CK2α-CK2β interaction is essential for the control of synapse stability

(A) A genomic region of Drosophila and human CK2α displaying CK2β-interaction and kinase domains (underlined). The residues required for CK2α-CK2β interaction (leucine 39 and phenylalanine 52 in Drosophila) are marked in red. Lysine 66 essential for CK2α kinase function is marked in blue. **(B)**

Immunoprecipitation (IP) assay for testing mutations abolishing CK2α-CK2β interaction. HA-tagged CK2β was efficiently precipitated by GFP-tagged CK2α or by GFP-tagged kinase-dead $CK2\alpha^{K66M}$. Modestly reduced amount of HA-CK2β was pulled down by GFP-$CK2\alpha^{L39A}$ or GFP-$CK2\alpha^{F52A}$. Very low level of HA-CK2β protein was precipitated by the double mutant GFP-$CK2\alpha^{L39A*F52A}$. As a negative control, an empty GFP construct was not able to precipitate HA-CK2β. **(C)** Western Blot analysis of larval brain extracts probed with the antibodies against CK2α, CK2β, and Tubulin. CK2α and CK2β protein levels were restored by ubiquitous expression of wild type CK2α, $CK2\alpha^{L39A}$ or $CK2\alpha^{F52A}$ in $CK2\alpha^{P2/P2}$ null animals. $CK2\alpha^{L39A*F52A}$ failed to rescue the loss of CK2β protein despite presence of equivalent CK2α protein levels. **(D)** Quantification of synaptic retraction frequency on muscles 1/9, 2/10. Significant differences between compared groups are noted by asterisks (***≤0.001, n=12-24 animals). Ubiquitous expression of CK2α, $CK2\alpha^{L39A}$, or $CK2\alpha^{F52A}$ in CK2α mutant background perfectly rescued synaptic stability, while expression of $CK2\alpha^{L39A*F52A}$ did not. Both CK2α mutants and animals rescued with $CK2\alpha^{L39A*F52A}$ showed a significant increase in synaptic retractions compared to control animals. **(E)** NMJs on muscle 4 stained for the green fluorescent protein (GFP, green), presynaptic vesicle domain marker DvGlut (blue), and the presynaptic motorneuron membrane marker Hrp (red). Scale bar: 5 μm. GFP-tagged wild type and kinase-dead CK2α and CK2β could efficiently localize to the presynaptic nerve terminal in either wild type or CK2α mutant background. As indicated by the GFP and Hrp staining, CK2α and CK2β were localized to synaptic bouton and inter-bouton regions and partially overlapped with synaptic vesicle domains marked by DvGlut.

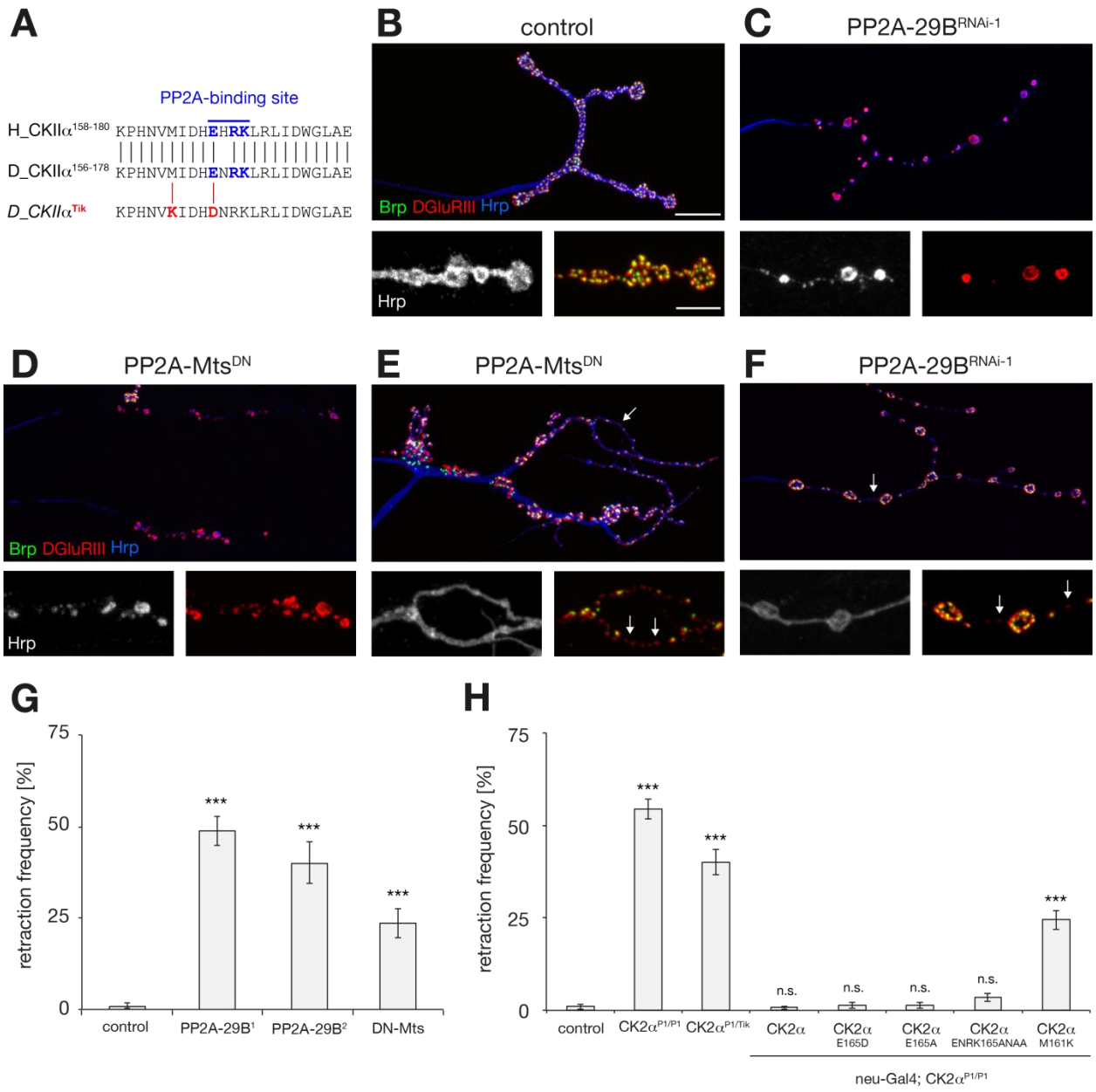
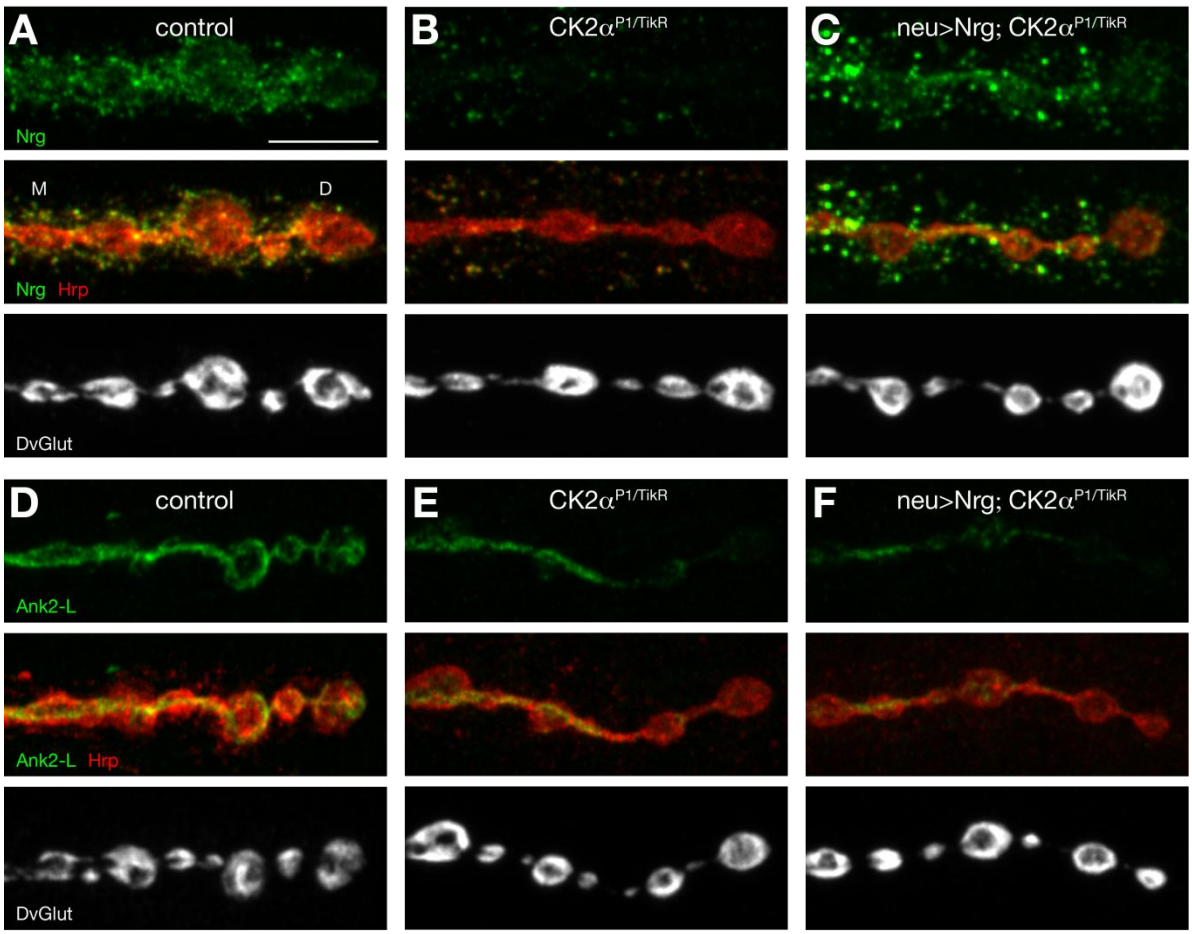


Figure 6 – CK2 controls synapse stability independently of PP2A

(A) A genomic region of human and Drosophila CK2α displaying a conserved PP2A-binding site (underlined). Three residues required for CK2α-PP2A interaction (glutamine 165, arginine 167, and lysine 168 in Drosophila) are marked in blue. Two amino acid substitutions in Drosophila Tik are marked in red (methionine 161 changed to lysine and glutamine 165 changed to aspartic acid). **(B-F)** NMJs on muscle 4 stained for the presynaptic active zone marker Brp (green), postsynaptic glutamate receptors DGluRIII (red), and the presynaptic motorneuron membrane marker Hrp (white). Scale bars: overviews 10 µm, insets 5 µm. **(B)** A stable wild type NMJ on muscle 4 is shown. **(C, D)** Knocking down presynaptic PP2A using RNAi (C) or abolishing PP2A function by expressing the dominant-negative catalytic subunit Mts (D) caused a severe retraction phenotype as indicated by the fragmentation of the

presynaptic membrane and the loss of presynaptic Brp. **(E)** Expression of the dominant-negative catalytic subunit Mts also resulted in a synapse formation defect indicated by long protrusion-like structures (arrow, overview) largely composed of glutamate receptor clusters not apposed with Brp (arrows, inset). The presynaptic membrane (Hrp) remained intact indicating a stable NMJ. **(F)** Knock down of presynaptic PP2A using RNAi caused a synaptic formation defect indicated by glutamate receptors unopposed by active zones (Brp) (arrows). **(G-H)** Quantification of synaptic retraction frequency on muscles 1/9, 2/10. Significant differences between compared groups are noted by asterisks (***≤0.001, n=9-24 animals). **(G)** Knocking down presynaptic PP2A using two independent RNAi lines or abolishing PP2A function by expressing the dominant-negative catalytic subunit Mts caused a significant increase in synaptic instability. **(H)** A synaptic retraction phenotype was rescued by presynaptically expressing CK2α, $CK2\alpha^{E165D}$, $CK2\alpha^{E165A}$, or $CK2\alpha^{ENRK165ANAA}$ in CK2α mutant background, whereas expressing $CK2\alpha^{M161K}$ was not sufficient for the complete phenotype rescue.


A
control
Nrg
M
D
Nrg Hrp
DvGlut
B
CK2α^P1/TikR
C
neu>Nrg; CK2α^P1/TikR
D
control
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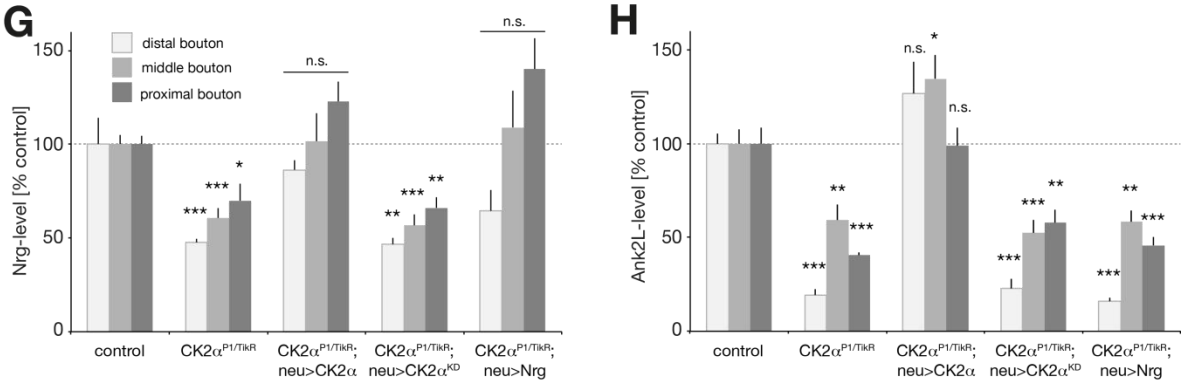
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middle bouton
proximal bouton
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150
100
50
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*
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CK2α^P1/TikR
CK2α^P1/TikR; neu>CK2α
CK2α^P1/TikR; neu>CK2α^KD
CK2α^P1/TikR; neu>Nrg

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Ank2L-level [% control]
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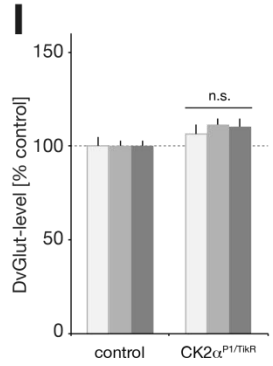
control
CK2α^P1/TikR
CK2α^P1/TikR; neu>CK2α
CK2α^P1/TikR; neu>CK2α^KD
CK2α^P1/TikR; neu>Nrg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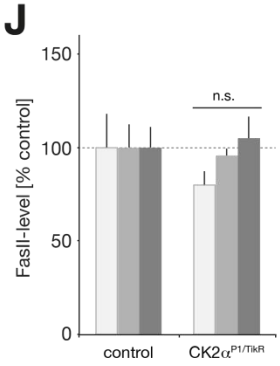
CK2α^P1/TikR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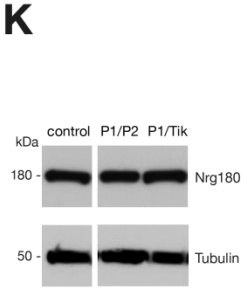
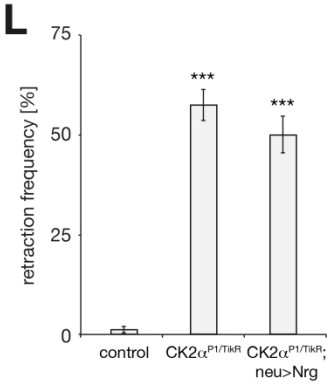
CK2α^P1/TikR


K
kDa
control
P1/P2
P1/Tik
180 -
Nrg180
50 -
Tubulin


L
retraction frequency [%]
75
50
25
0
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control
CK2α^P1/TikR
CK2α^P1/TikR; neu>Nrg

Figure 7 – CK2 maintains the synaptic localization of essential synaptic proteins

(A-C) Muscle 4 NMJs stained with an antibody specific to the cytoplasmic tail of the cell adhesion molecule Neuroglian (Nrg, green), the presynaptic motorneuron membrane marker Hrp (red), and the presynaptic vesicle domain marker DvGlut (white). Examples shown here are stable synapses indicated by the presence of the presynaptic vesicle domains (DvGlut) and the continuous presynaptic membrane (Hrp). Scale bar: 5 μm. **(A)** In wild type animals Nrg was localized within the presynaptic nerve terminal marked by the membrane marker (Hrp). Nrg was distributed in a punctuate pattern surrounding synaptic vesicle domain clusters and co-localizing with Hrp at the ends of small membrane extensions. The Nrg localization pattern and intensity appeared uniform throughout the presynaptic nerve terminal. The most distal bouton is marked with 'D'. **(B)** In CK2α mutants we observed a substantial reduction in the Nrg staining intensity throught the entire nerve terminal but especially in the most distal bouton regions. **(C)** Neuronal expression of Nrg in CK2α mutants was sufficient to restore presynaptic Nrg levels as indicated by the re-appearance of Nrg staining. **(D-F)** Muscle 4 NMJs stained with an antibody specific to the long isoform of the adaptor molecule Ankyrin-2 (Ank2-L, green), the presynaptic motorneuron membrane marker Hrp (red), and the presynaptic vesicle domain marker DvGlut (white). Examples shown here are stable synapses indicated by the presence of the presynaptic vesicle domains (DvGlut) and the continuous presynaptic membrane (Hrp). Scale bar: 5 μm. **(D)** In wild type animals Ank2-L was present uniformly within boutons and inter-bouton regions in the presynaptic nerve terminal. **(E)** In CK2α mutants we observed a reduction in the Ank2-L staining intensity which was especially pronounced in the most distal bouton regions. **(F)** Neuronal expression of Nrg in CK2α mutants was not sufficient to restore presynaptic Ank2-L levels. The Ank2-L localization and intensity patterns were indistinguishable from (E). **(G-J)** Quantification of presynaptic Nrg (G), Ank2-L (H), DvGlut (I), and FasII (J) intensity levels, measured in the most distal (white bars), middle (grey bars), and proximal (dark grey bars) boutons of a given NMJ branch using Imaris (Bitplane). DvGlut staining was used to define distal, middle, and proximal boutons of the presynaptic nerve terminal. Proximal bouton is defined as the bouton situated closest toward the site where a given NMJ branches out. Distal bouton is defined as the bouton situated at the terminal of a given NMJ (as in 7A, bouton marked with letter 'D'). The middle bouton is situated right in the middle between proximal and distal boutons. The staining fluorescence intensity values are normalized to the appropriate wild type controls. Significant differences between compared groups are noted by asterisks (* ≤0.05, **≤0.01, ***≤0.001, segment A4, muscle 4, n=6-12 NMJs). **(G)** In CK2α mutants the Nrg levels were significantly reduced in distal, middle, and proximal boutons compared to wild type controls. Presynaptic expression of wild type CK2α or Nrg, but not kinase-dead CK2α, was sufficient to restore Nrg levels back to normal. **(H)** In CK2α mutants Ank2-L levels were significantly reduced in distal, middle, and proximal boutons compared to wild type controls. The most severe reduction was observed in most distal boutons. Presynaptic expression of wild type CK2α, but not Nrg or kinase-dead CK2α, was sufficient to restore Nrg levels. **(I-J)** Presynaptic levels of DvGlut (I) or FasII (J) were indistinguishable in wild type and CK2α mutant animals. **(K)** Western Blot analysis of larval brain extracts probed with an antibody against Nrg180 and Tubulin. CK2α mutants ($CK2\alpha^{P1/P1}$ and $CK2\alpha^{P1/Tik}$) showed the same Nrg protein levels as the wild type control. **(L)** Quantification of retraction frequency on muscles 1/9, 2/10. Neuronal expression of Nrg in CK2α mutant animals failed to rescue the impairment in synapse stability. Both CK2α mutants and CK2α mutants neuronally expressing Nrg showed a significant increase in synaptic retraction frequency compared to wild type animals (P≤0.001, n=9-15 animals).

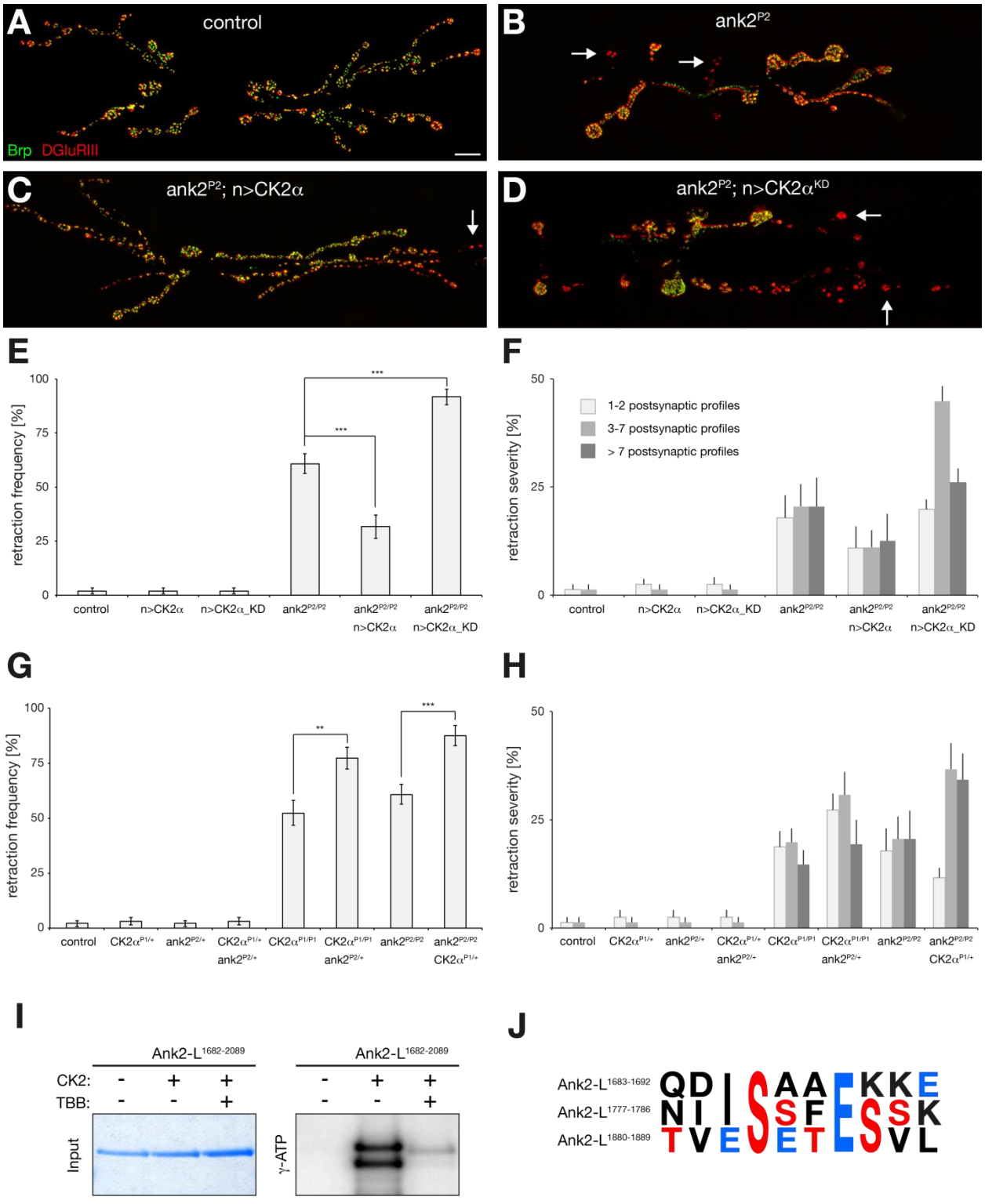

Figure 8 – CK2 controls synaptic stability via Ank2-L

(A-D) NMJs on muscle 6/7 stained for the presynaptic active zone marker (Brp, green) and postsynaptic glutamate receptors (DGluRIII, red). Scale bar: 10 µm. **(A)** A stable wild type NMJ indicated by a perfect

apposition of pre- and postsynaptic markers. **(B)** In Ank2 mutant animals, we observed synaptic retractions indicated by the presence of fused glutamate receptor clusters lacking the apposing Brp (arrows). **(C)** Expressing wild-type CK2α in Ank2 mutants partially, but not fully, rescued a synaptic retraction phenotype. The synaptic retraction is indicated by an arrow. **(D)** Expressing kinase-dead CK2α in Ank2 mutants further enhanced synaptic instability defects. Retractions of multiple synaptic branches are indicated by arrows. **(E, G)** Quantification of synaptic retraction frequency on muscle 6/7. Significant differences between compared groups are noted by asterisks (**≤0.01, ***≤0.001, n=12-15 animals). **(E)** Expressing wild type or kinase-dead CK2α in wild type background did not lead to a significant increase in synaptic retraction frequency compared to wild type control. Expressing kinase-dead CK2α in Ank2 mutant animals significantly increased while expressing wild type CK2α in Ank2 mutants significantly decreased the frequency of synaptic retractions. **(G)** CK2α and Ank2 genetically interact. We did not observe a significant change in the frequency of retractions in trans-heterozygous $CK2\alpha^{P1/+}$ $Ank2\text{-}L^{P2/+}$ animals compared to wild type control. Removal of one copy of Ank2 in CK2α mutant animals and removal of one copy of CK2α in Ank2 mutant animals significantly increased the frequency of synaptic retractions compared to CK2α and Ank2 mutants respectively. **(F, H)** Quantification of retraction severity on muscle 6/7 of the same genotypes as in (E) and (G). A trend towards the enhancement of retraction severity was observed in Ank2 mutants neuronally expressing kinase-dead CK2α (F, last column) and Ank2 mutants in which one copy of CK2α was removed (H, last column). **(I)** In vitro phosphorylation analysis using Ank2-L microtubule-binding domain ($Ank2\text{-}L^{1682\text{-}2089}$) and γ-ATP as substrates for purified human CK2. CK2 kinase and CK2-spedific inhibitor TBB were added or not as indicated by '+' or '-' respectively. CK2 was able to efficiently phosphorylate $Ank2\text{-}L^{1682\text{-}2089}$ only in the absence of TBB as indicated by the presence of a strong radioactive signal. **(J)** Three Ank2-L residues identified by mass spectroscopy to be efficiently phosphorylated by CK2 are indicated (S1686, S1780, S1883 indicated as capital 'S' in red). All three serine residues reside within the CK2 phosphorylation consensus sequence SXXE.

Supplemental Table 1

VDRC Kinase List: 313 kinases, kinase-like proteins, and subunits screened

Name	CG	VDRC number
Abl	CG4032	2897
AC 78C	CG10564	51978
AC e135	CG12243	45641
AC76E	CG7978	51975
AC 13E	CG9210	11547
AC3	CG1506	33217
Ack e139	CG14992	39857
AC e163	CG8970	12239
ACXA	CG17176	49100
ACXB	CG17174	9748
ACXC	CG5983	2870
alk	CG8250	11446
apl	CG15509	6212
ATM e148	CG6535	22502
atyp abu	CG4410	26534
atyp PIK	CG2905	52486
auxillin	CG1107	16182
ball	CG6386	48980
BCR e115	CG17960	33030
bon	CG5206	44284
bsk jnk	CG5680	34138
bt projectin	CG32019	46252
btk29A	CG8049	22675
btl	CG32134	27106
Bub1	CG7838	26109
BUB1 like	CG14030	24833
Cad96Ca	CG10244	1089
CAMKII	CG18069	47280
Caki	CG6703	34184
Cdc2	CG5363	41838
		106130
CDC7	CG5790	45045
CDC7related e40	CG8655	40715
cdi	CG6027	43634
Cdk4	CG5072	40577
Cdk5	CG8203	35855
Cdk7	CG3319	10442

Cdk8	CG10572	31264
Cdk9	CG5179	30448
Cdk12	CG7597	25508
CG10673	CG10673	27301
CG11228	CG11228	7823
CG11660	CG11660	18526
CG14163	CG14163	5199
CG14305	CG14305	17477
CG17698	CG17698	35632
CG1951	CG1951	33431
CG2577	CG2577	41693
		41694
		105471
CG3008	CG3008	52634
CG3105	CG3105	25661
CG4041	CG4041	34780
CG4290	CG4290	26496
CG43143	CG43143	16334
CG6800	CG6800	40394
CG7156	CG7156	26035
CG7177	CG7177	35193
CG7616	CG7616	41408
CG8173	CG8173	35845
CG8726	CG8726	40719
CG9222	CG9222	27010
CG9818	CG9818	16074
CHED-related	CG7497	9374
CKIα	CG2028	13664
CKIαlike	CG7097	27843
Ckαlike 6e-78	CG9962	36473
		36178
		108721
		108273
Ckαlike e103	CG12147	31658
		31659
		101875
CKβ	CG15224	32377
		32378

		26914
		26915
		102633
CK2β1	CG13591	17282
CKI like	CG11533	45121
csk	CG17309	32877
dco	CG2048	9241
ddr	CG11573	51719
DGKβ	CG18654	38239
		38240
DGKE	CG8657	4659
DGK e126	CG31452	5097
DGK e159	CG9851	43739
dlk pk e138	CG8789	26910
dnt	CG17559	6547
doa	CG1658	19066
dome	CG14226	19717
drak	CG1760	32961
drl	CG17348	3047
Drl2	CG3915	40484
Dsor1	CG15793	40026
egfr	CG10079	4326
eip63E	CG10579	47860
emk	CG15072	39864
eph	CG4371	4771
ERK7 4e-96	CG2309	13444
Ethanolamine kinase	CG3525	34286
Fak56D	CG10023	17957
for	CG10033	38319
fps85D	CG8874	36053
fray	CG7693	41718
fs(1)h	CG2252	51227
		108662
fu	CG6551	27663
FYVE	CG6355	27592
Gck III	CG5169	49559
gek	CG4012	28367
gish	CG6963	26003
		106826
GLK	CG7097	35166
grp	CG17161	12680

		110076
GSK 3β	CG11338	25640
gwl	CG 7719	21046
GYC 0.0	CG3216	29915
Gyc88E	CG4154	21797
GYC 2e-71	CG5719	20107
GYC 32E	CG6275	845
GYC 76C	CG8742	3058
GYCβ 1e-92	CG14885	46916
GYC e165	CG9783	29276
hep	CG4353	47509
Hipk	CG17090	32854
hop	CG1594	40037
htl	CG7223	27180
ial	CG6620	35107
ik2	CG2615	12485
inaC	CG6518	2895
inR	CG18402	991
		992
IRE like e112	CG4583	39561
Ird5	CG4201	26427
KP78a	CG6715	51616
KP78b	CG17216	51995
ksr	CG2899	45040
LIMK	CG1848	25343
Lrrk	CG5483	22139
Madm	CG1098	27346
mak	CG4588	25813
mapkap	CG3086	3170
MAST205 e140	CG6498	35100
		35101
		109282
mei41	CG4252	11251
mekk1	CG7717	25529
mkk4	CG9738	26929
MLCK like	CG1776	44737
MLCK like e32	CG10177	38349
mnb	CG7826	28628
		107066
Mos e23	CG8767	36531
MPSK	CG1227	38647

Myt1	CG32417	34547
Nak	CG10637	35482
Nek2	CG17256	40052
NEK (6e-59)	CG10951	16120
Nina C	CG54125	27360
nmo	CG7892	3002
ok	CG10895	44980
otk	CG8967	30833
pII	CG5974	2889
pak	CG10295	12553
Pak3	CG14895	39844
Par1	CG8201	52553
PDK	CG8808	37966
PEK	CG2087	16427
PhKgamma	CG1830	33054
phl	CG2845	20909
PI3K68D	CG11621	16240
PI3K92E	CG4141	38985
PI4k 0.0	CG10260	15993
		105614
PI4K 2e-80	CG7004	27786
Pink1	CG4523	21860
PIP5K	CG9985	6229
PIP5K59B	CG3682	47027
pitslre	CG4268	45127
pk34A	CG5182	27368
PK61C	CG1210	18736
pk92B	CG4720	34891
PKA like	CG12069	23719
PKA-C1	CG4379	101524
PKA C2	CG12066	30658
PKA-RI	CG42341	103720
PKA-RIII	CG15862	101763
PKC53E	CG6622	27699
PKC98E		33434
PKC delta	CG10524	31468
		22755
		33837
		33838
		101421
PKE like	CG4839	26641
PK17E	CG7001	26006

Pkg21D	CG3324	34594
png	CG11420	31500
polo	CG12306	20177
Prp4	CG 7028	27808
pvr	CG8222	43459
Rack1	CG7111	27858
		27859
RDGK	CG10966	3024
ret	CG14396	843
rok	CG9774	3793
ror	CG4926	935
R-PTK 6e-94	CG7180	34369
rtk	CG3277	7271
rutabaga	CG9533	5569
		101759
SAK	CG7186	27904
SAX	CG1891	9434
sev	CG18085	49924
sgg	CG2621	7005
shark	CG18247	25304
S6K	CG10539	18126
s6kII	CG17596	5702
SLK e110	CG4527	43783
slpr	CG2272	33516
smi35A	CG4551	40534
snf1A	CG3051	1827
SNF1related	CG6114	22225
SNRK	CG8485	35940
sphingosine kinase1	CG1747	32932
sphingosine kinase2	CG2159	34565
Src42A	CG7873	26019
SRPK	CG8174	26933
SRPK2 3e-77	CG8179	35848
SRPK79D	CG11489	47544
Src64B	CG7524	35252
Ste20	CG18582	46043
Taf250	CG17603	41099
takl1	CG31421	25760
Takl2	CG4803	34898

Tao1 e146	CG14217	17432
tkv	CG14026	3059
tor	CG1389	4298
trbl	CG5408	22114
UNC 51 like atg1	CG10967	16133
unc-89	CG18021	29412
VRK	CG8878	28970
wee	CG4488	26543
wit	CG10776	865
yata	CG1973	19275
yu/PKA anchor protein	CG3249	48005
		48006

VDRC Phosphatase List: 127 phosphatases and subunits screened

Name	CG number	VDRC number
Acph-1	CG7899	3579
Aph4	CG1462	6862
atyp STP	CG3376	12226
CanA1	CG1455	32283
CanB2	CG11217	28762
CamA-14F	CG9819	30105
CDC14 e124	CG7134	27881
CG11426	CG11426	42599
CG14411	CG14411	17576
CG15035	CG15035	32353
CG15528	CG15528	14865
CG15533	CG15533	42520
CG15534	CG15534	30186
CG16717	CG16717	29199
CG17026	CG17026	49199
CG17029	CG17029	32823
CG17598	CG17598	32956
CG17746	CG17746	40102
CG3980	CG3980	34773
CG5026	CG5026	34915
CG9236	CG9236	39162
CSW	CG3954	21756
eya	CG9554	43911
fig pp2 cat act	CG7615	47312
flfl	CG9351	24143

		103793
flw	CG2096	29622
fragment PTP TD14	CG9311	14173
homology to PIR 4e-16	CG13197	36422
l-2	CG10574	39053
IMP 1e-46	GC17027	50075
IMP 1e-66	CG939	23723
IMP 5e-32	CG17028	32819
IMP 5e-47	CG9389	44663
IPP	CG3028	25615
IPP 2e-60	CG9784	30098
IPP 2e-82	CG7613	25030
IPP 3e-50	CG9801	104525
IPP 9e-35	CG10426	16048
IPP CG6805	CG6805	107728
IPP synaptojanin	CG6562	46070
l-t	CG14719	32316
LAR	CG10443	36269
laza	CG11440	42592
LMW PTP 3e-19	CG14297	102071
mapmodul	CG5784	49385
mbs	CG32156	105762
meg1 (e 144)	CG1228	38651
Meg2	CG3101	33744
		21275
		21276
		104427
Mipp1	CG4123	8493
Mipp2		14163
		108018
mkp	CG34099	104374
Mkp3	CG14080	45415
MKP like	CG14211	3146
MKP like 3e-25	CG7378	35226
MKP like PUC 1e-18	CG10089	17991
Mppe	CG8889	47953
Mre11	CG16928	30474
mts	CG7109	35171
myotubularin	CG9115	29032
myotubularin like	CG3632	26254
myotubularin like e164	CG3530	26217
NipP1	CG8980	42175
ocrl	CG3573	34649

pdp	CG12151	31661
Plip	CG10371	47623
pp1 87B	CG5650	35025
PP1-13C	CG9156	29058
		29057
		107770
ppD3	CG8402	24308
ppD5	CG10138	18016
PPI inh act	CG17124	19078
PPI inh act	CG6380	29950
PP1 regulatory activity	CG13125	17123
PP1 reg act	CG9619	36121
pp1 reg act sds22	CG5851	42051
pp2a reg act	CG32568	46651
PP1 reg act	CG9238	24150
PP2A-B'	CG7913	22614
PP2A-29B reg subunit	CG17291	49671
		49672
		23886
PP2B-14D	CG9842	46873
pp2b reg act canB	CG4209	21611
Pp2C1	CG2984	33599
PP2C 7e-35	CG7115	9404
PP2Cβ e101	CG6036	21023
PP2Cβ e110	CG1906	32476
PP2C cat act	CG12091	13985
PP4-19C	CG18339	25317
PP4 like	CG11597	38540
		104729
PP4R2	CG2890	105399
		25445
Ppm1	CG12169	38631
ppn58A	CG3245	41901
PPY55A	CG10930	16096
PPYRI	CG15031	18506
PRL	CG4993	21558
Primo-1	CG31469	23397
PTEN	CG5671	35731
PTP activated activity	CG2104	30018
PTP activator act	CG8509	28915
PTP cat act PIP52F	CG18243	3116
PTPD1	CG9493	40743
ptp99A	CG11516/2005	27207
ptp10D	CG1817	1102

ptp69D	CG10975	27090
ptp4E	CG6899	1012
ptp61F	CG9181	37436
PTP-like catalytic activity	CG6746	46513
PTPN6 5e-27	CG14714	47455
PUC MKP	CG7850	3018
RDGC	CG6571	35105
R-PTP99A	CG2005	6705
R-PTPX	CG4355	7560
sbf	CG6939	22317
set	CG4299	21827
sim to PTP	CG92671	2879
spn	CG16757	19658
		105888
		107428
ssh	CG6238	30136
stg	CG1395	17760
Tie	CG7525	27087
twine	CG4965	46064
tws	CG6235	34340
wdb	CG5643	27470
WUN	CG8804	6446
WUN-like	CG8805	4176
WUNlike 1e-31	CG11437	9452
WUN like 4e-40	CG11425	8458
wunlike PAP2A	CG12746	33313
yrt	CG9764	28674

Supplemental Table 2

RNAi Screen

RNAi		Retraction frequency (%)						
UAS	Gal4	M 1/9, 2/10	p	M 6/7	p	M 4	p	n
Wild type	Elav; dic2	1.0±0.7		2.1±1.4		2.1±1.4		12
CK2β VDRC32377	Elav; dic2	51.4±4.0	1.69E-06	20.8±6.6	0.021279	18.3±5.6	0.012127	9
PP2A-29B[1] VDRC49671	Elav; dic2	48.7±3.9	1.05E-08	64.3±5.2	7.62E-09	56.7±3.9	1.21E-09	14
PP2A-29B[2] BL29384	Elav; dic2	40.0±5.6	7.1E-05	47.1±7.8	7.27E-05	27.9±5.1	0.000258	10
IPP CG9784 VDCR30098	Elav; dic2	35.6±7.3	0.001135	25.0±7.9	0.01713	16.3±4.6	0.013086	10
CKIα[1] VDRC13664	Elav; dic2	33.3±4.9	4.65E-05	19.4±4.7	0.006391	27.8±6.2	0.002846	12
CKIα[2] BL25786	Elav; dic2	25.0±3.9	0.000191	16.7±4.4	0.007965	28.1±3.8	1.61E-05	9
Mnb[1] VDRC35282	Elav; dic2	22.9±5.5	0.004315	10.8±3.0	0.014565	10.0±2.2	0.005673	9
Mnb[2] VDRC107066	Elav; dic2	9.6±1.6	0.000101	13.9±5.3	0.059316	8.3±3.0	0.079648	15
MAST205 VDRC35100	Elav; dic2	19.8±3.3	0.000137	8.3±3.6	0.124167	11.5±4.5	0.066975	12
PI4KIIIα[1] VDRC15993	Elav; dic2	12.5±2.6	0.001906	5.6±2.2	0.204143	11.1±5.3	0.133384	9
PI4KIIIα[2] VDRC105614	Elav; dic2	8.3±2.1	0.007792	2.8±1.8	0.767835	6.9±3.0	0.173138	9
PP4-19C VDRC25317	Elav; dic2	12.5±2.6	0.000817	2.1±2.1	1.0	16.7±3.9	0.003262	12
inR VDRC991	Elav; dic2	10.4±1.8	0.000679	6.9±3.0	0.173138	9.7±4.6	0.140024	9
Mipp2 VDRC14163	Elav; dic2	7.6±1.4	0.00115	8.3±2.9	0.079648	11.1±3.3	0.027233	9
Cdc2 VDRC41838	Elav; dic2	6.9±1.6	0.006733	5.6±3.0	0.32043	5.6±2.2	0.204143	9

CK2β rescues and MARCM

Genotype	Retraction frequency (%) m 1/9, 2/10	p	n

Wild type	1.0±0.7		12
Neu>CK2β RNAi	51.4±4.0	1.69E-06	9
Muscle>CK2β RNAi	1.4±0.9	0.767835	9
Neu>CK2β RNAi; neu>CK2β	3.8±1.4	0.1025	10
Neu>CK2β RNAi; neu>GFP	33.9±2.8	9.59E-08	12
$CK2\beta^{26\text{-}2}$/mbuP1	13.8±3.0	0.002341	10
$CK2\beta^{26\text{-}2}$; ubi>CK2β	2.9±1.1	0.185102	13

Genotypes		p
Neu>CK2β RNAi	Neu>CK2β RNAi; neu>CK2β	5.37E-07
Neu>CK2β RNAi	Neu>CK2β RNAi; neu>GFP	0.00274

Genotype	% of retractions on muscles innervated by GFP-positive neurons	p	n
MARCM control	0.0±0.0		17
MARCM $CK2\beta^{26\text{-}2}$	29.0±5.0	7.24E-06	33

CK2α alleles

Allele combination		Retraction frequency (%)				
		M 1/9, 2/10	p	M6/7	p	n
Wild type		1.0±0.7		2.1±1.7		12
$CK2\alpha^{P1}$	$CK2\alpha^{P1}$	57.5±3.9	4.22E-10	53.3±7.4	5.66E-06	15
$CK2\alpha^{P1}$	$CK2\alpha^{P2}$	33.0±3.8	8.84E-07	23.2±4.7	0.000621	14
$CK2\alpha^{P1}$	$CK2\alpha^{Tik}$	40.1±3.4	7.34E-11	33.0±5.0	3.62E-06	22
$CK2\alpha^{P1}$	$CK2\alpha^{TikR}$	54.0±5.0	7.5E-09	44.9±6.0	1.65E-06	17
$CK2\alpha^{P1}$	$CK2\alpha^{G703}$	36.1±2.9	9.32E-07	27.8±7.1	0.006451	9
$CK2\alpha^{P1}$	$CK2\alpha^{H3091}$	32.8±4.6	1.68E-05	18.6±4.5	0.003586	12
$CK2\alpha^{P2}$	$CK2\alpha^{P2}$	Embryonic lethal				
$CK2\alpha^{P2}$	$CK2\alpha^{Tik}$	31.8±2.1	1.5E-17	19.1±2.5	3.85E-07	37
$CK2\alpha^{P2}$	$CK2\alpha^{TikR}$	Embryonic lethal				
$CK2\alpha^{P2}$	$CK2\alpha^{G703}$	38.6±4.1	2.07E-06	35.2±4.1	5.51E-06	11
$CK2\alpha^{P2}$	$CK2\alpha^{H3091}$	21.9±3.1	2.92E-05	12.5±4.4	0.040278	12
$CK2\alpha^{Tik}$	$CK2\alpha^{Tik}$	Embryonic lethal				
$CK2\alpha^{Tik}$	$CK2\alpha^{TikR}$	Embryonic				

		lethal				
$CK2\alpha^{Tik}$	$CK2\alpha^{G703}$	30.2±4.9	9.66E-05	17.7±3.9	0.002108	12
$CK2\alpha^{Tik}$	$CK2\alpha^{H3091}$	12.1±2.0	6.2E-05	10.9±3.0	0.014054	16
$CK2\alpha^{TikR}$	$CK2\alpha^{TikR}$	Embryonic lethal				
$CK2\alpha^{TikR}$	$CK2\alpha^{G703}$	51.4±5.4	1.5E-05	47.2±6.2	5.48E-05	9
$CK2\alpha^{TikR}$	$CK2\alpha^{H3091}$	29.9±4.0	0.000103	15.3±4.0	0.01167	9
$CK2\alpha^{G703}$	$CK2\alpha^{G703}$	Embryonic lethal				
$CK2\alpha^{G703}$	$CK2\alpha^{H3091}$	17.4±3.0	0.000115	15.2±2.7	0.000361	14
$CK2\alpha^{H3091}$	$CK2\alpha^{H3091}$	Embryonic lethal				

CK2α rescues

Genotype	Retraction requency (%) M 1/9, 2/10	p	n
Wild type	1.1±0.8		12
$CK2\alpha^{P1/P1}$	57.5±3.9	4.22E-10	15
$CK2\alpha^{P1/P2}$	33.0±3.8	8.84E-07	14
$CK2\alpha^{P1/Tik}$	40.1±3.4	7.34E-11	22
$CK2\alpha^{P1/TikR}$	54.0±5.0	7.5E-09	17
$CK2\alpha^{P1/P1}$ neu>CK2α	1.0±0.7	0.92807	12
$CK2\alpha^{P1/P1}$ neu>CK2α_KD	65.8±.9	2.49E-14	17
$CK2\alpha^{P1/P1}$ muscle>CK2α	52.5±3.3	1.49E-10	15
$CK2\alpha^{P1/P1}$ ubi>CK2α	0.7±0.5	0.665861	17
$CK2\alpha^{P2/P2}$; ubi>CK2α elavGal80	41.7±4.3	6.57E-06	9

Genotypes		p
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}$ neu>CK2α	4.22E-10
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}$ neu>CK2α_KD	0.099286
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}$ muscle>CK2α	0.335885
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}$ ubi>CK2α	8.88E-10

DvGlut cluster number

Genotype	DVGLUT cluster number on m4, %	p	N (NMJs)

Wild type	100±4.0		48
$CK2\alpha^{P1/TikR}$	40.8±2.8	1.11E-20	48
$CK2\alpha^{P1/P1}$ neu>CK2α	99.9±4.2	0.985266	48
$CK2\alpha^{P1/P1}$ neu>CK2α_KD	38.0±2.5	6.93E-22	48
Neu>CK2α/CKIIα	170.9±5.5	7.89E-17	48

Genotypes		p
$CK2\alpha^{P1/TikR}$	$CK2\alpha^{P1/P1}$ neu>CK2α	3.76E-19
$CK2\alpha^{P1/TikR}$	$CK2\alpha^{P1/P1}$ neu>CK2α_KD	0.429068

Satellite bouton number

Genotype	Satellite bouton number on m4, average	p	N (NMJs)
Wild type	0.6±0.2		20
neu>CK2α/CK2α	20.5±2.4	1.38E-07	20

Rescues with different CK2α mutants

Genotypes	Retraction frequency (%)		
	M 1/9,2/10	p	n
Wild type	1.0±0.7		12
$CK2\alpha^{P1/P1}$	54.4±2.8	1.17E-16	24
$CK2\alpha^{P1/Tik}$	40.1±3.4	7.34E-11	22
$CK2\alpha^{P1/P1}$ ubi>CK2α	0.7±0.5	0.726453	17
$CK2\alpha^{P1/P1}$ ubi>CK2α_E165D	1.4±0.9	0.767835	9
$CK2\alpha^{P1/P1}$ ubi>CK2α_E165A	1.4±0.9	0.767835	9
$CK2\alpha^{P1/P1}$ ubi>CK2α_ERKAAA	3.5±1.1	0.083311	9
$CK2\alpha^{P1/P1}$ ubi>CK2α_M161K	24.5±2.6	8.94E-07	12
$CK2\alpha^{P1/P1}$ ubi>CK2α_L39A	1.6±0.7	0.684095	16
$CK2\alpha^{P1/P1}$ ubi>CK2α_F52A	2.7±1.1	0.25442	16
$CK2\alpha^{P1/P1}$ ubi>CK2α_L39AF52A	19.9±3.4	3.64E-05	17

Genotypes		M 1/9, 2/10, p
$CK2\alpha^{P1/Tik}$	$CK2\alpha^{P1/P1}$ ubi>CK2α_M161K	0.000928
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}$ ubi>CK2α_L39AF52A	2.79E-09

PP2A-CK2 genetic interaction

RNAi		Retraction frequency (%)						
UAS	Gal4	M 1/9, 2/10	p	M 6/7	p	M 4	p	n
Wild type	Elav; dic2	1.0±0.7		2.1±1.4		2.1±1.4		12
$PP2A\text{-}29B^{1}$ VDRC49671	Elav; dic2	48.7±3.9	1.05E-08	64.3±5.2	7.62E-09	56.7±3.9	1.21E-09	14
$PP2A\text{-}29B^{2}$ BL29384	Elav; dic2	40.0±5.6	7.1E-05	47.1±7.8	7.27E-05	27.9±5.1	0.000258	10
$PP2A\text{-}29B^{1}$ VDRC49671 Early 3d instar	Elav; dic2	7.3±2.4	0.027224	4.2±2.3	0.456578	6.3±3.3	0.259241	12
DN-Mts	Elav; sca	23.6±4.1	0.000112	10.6±2.8	0.013737	15.4±4.9	0.021277	13

Genotypes	Retraction frequency (%)						
	M 1/9, 2/10	p	M 6/7	p	M 4	p	n
Wild type	1.0±0.7		2.1±1.4		2.1±1.4		12
$PP2A^{GE16781/+}$	3.1±1.2	0.15539	1.6±1.6	0.807368	0.0±0.0	0.166087	9
$CK2\alpha^{P1/+}$	1.0±0.7	1.0	2.1±1.4	1.0	2.1±1.4	1.0	12
$PP2A^{GE16781/+}$; $CK2\alpha^{P1/+}$	3.5±1.5	0.173138	0.0±0.0	0.166087	0.0±0.0	0.166087	9
$CK2\alpha^{P1/P1}$	58.2±3.2	2.27E-12	52.4±5.7	2.89E-07	41.7±9.5	0.009163	19
$PP2A^{GE16781/+}$; $CKII\alpha^{P1/P1}$	46.0±3.6	6.34E-09	29.8±3.3	9.55E-07	20.2±3.3	0.000128	14

Genotypes		M 1/9, 2/10 p	M 6/7 p	M 4 p
$CK2\alpha^{P1/P1}$	$PP2A^{GE16781/+}$; $CK2\alpha^{P1/P1}$	0.015229	0.004586	0.076906

Nrg, Ank2, FasII, DvGlut, and Hrp levels

Genotypes	**Nrg-level (% control)**						
	Distal bouton	p	Middle bouton	p	Proximal bouton	p	n
Wild type	100.0±14.0		100.0±5.1		100.0±4.6		6
$CK2\alpha^{P1/TikR}$	47.5±2.0	0.013809	60.7±5.3	0.000484	70.0±9.0	0.024828	6
$CK2\alpha^{P1/TikR}$; neu>CKIIα	86.4±5.2	0.398193	101.7±15.1	0.919937	122.7±10.9	0.127887	6
$CK2\alpha^{P1/TikR}$; neu>CK2α_KD	46.7±3.4	0.010084	56.8±5.7	0.000308	66.2±5.7	0.001693	6
$CK2\alpha^{P1/TikR}$; neu>Nrg	64.5±11.4	0.080597	109.1±19.6	0.671471	140.0±16.6	0.068241	6
	Ank2L-level (% control)						
Wild type	100.0±5.3		100.0±7.6		100.0±8.4		6
$CK2\alpha^{P1/TikR}$	19.4±3.0	9.99E-07	59.1±8.3	0.00476	40.5±1.7	0.000945	6
$CK2\alpha^{P1/TikR}$; neu>CK2α	126.8±16.8	0.178839	134.7±12.6	0.046746	98.7±9.8	0.922847	6
$CK2\alpha^{P1/TikR}$; neu>CK2α_KD	22.6±5.1	9.65E-07	52.3±6.9	1.47E-05	57.7±7.1	0.003219	6
$CK2\alpha^{P1/TikR}$; neu>Nrg	15.9±2.1	5.76E-06	58.3±5.9	0.001979	45.8±4.3	0.000693	6
	Ank2XL-level (% control)						
Wild type	100.0±4.3		100.0±11.0		100.0±8.3		6
$CK2\alpha^{P1/TikR}$	18.3±1.8	4.81E-07	64.9±10.4	0.284101	55.7±3.2	0.087285	6
$CK2\alpha^{P1/TikR}$; neu>CK2α	91.9±11.4	0.531622	85.8±7.3	0.961498	69.5±11.7	0.393111	6
$CK2\alpha^{P1/TikR}$; neu>CK2α_KD	19.5±3.6	5.49E-08	39.0±7.0	0.027211	73.4±6.8	0.452136	6
$CK2\alpha^{P1/TikR}$; neu>Nrg	14.7±1.8	3.52E-07	57.0±3.8	0.118155	69.5±8.4	0.351639	6
	DVGLUT-level (% control)						
Wild type	100.0±4.8		100.0±2.8		100.0±2.9		12
$CK2\alpha^{P1/TikR}$	106.4±5.1	0.368138	111.4±3.3	0.015368	110.4±4.2	0.056714	12
	FasII-level (% control)						
Wild type	100.0±18.0		100.0±12.4		100.0±10.8		6
$CK2\alpha^{P1/TikR}$	80.1±7.1	0.351407	95.6±3.8	0.747492	104.9±11.5	0.76361	6
	HRP-level (% control)						
Wild type	100.0±3.6		100.0±5.0		100.0±3.9		12
$CK2\alpha^{P1/TikR}$	44.8±1.5	4.9E-10	47.6±2.2	8.29E-08	50.3±2.0	4.65E-09	12

Genotype	% retractions on m 1/9, 2/10	p	n
Wild type	1.1±0.7		12
$CK2\alpha^{P1/P1}$	57.5±3.9	4.49E-10	15
$CK2\alpha^{P1/P1}$; neu>Nrg	50±4.7	6.55E-06	9

Genotypes	% retractions on m 1/9, 2/10	p
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}$; neu>Nrg	0.233614

Ank2-CK2 genetic interaction

Genotype	Retraction frequency (%)				
	M 6/7	p	M 1/9, 2/10	p	n
Wild type	2.1±1.4		1.0±0.7		12
N>CK2α	2.1±1.4	1.0	1.0±0.7	1.0	12
N>CK2α_KD	2.1±1.4	1.0	1.6±0.8	0.633323	12
$Ank2^{P2/P2}$	60.8±4.5	6.43E-10	65.1±4.2	4.24E-09	15
$Ank2^{P2/P2}$ N>CK2α	31.7±5.4	0.000111	45.8±6.2	1.85E-05	13
$Ank2^{P2/P2}$ N>CK2α_KD	91.7±3.6	1.23E-12	86.4±4.0	3.37E-10	12

Genotypes		M 6/7, p	M 1/9, 2/10, p
$Ank2^{P2/P2}$	$Ank2^{P2/P2}$ N>CK2α	0.00039	0.019175
$Ank2^{P2/P2}$	$Ank2^{P2/P2}$ N>CK2α_KD	1.53E-05	0.001555
$Ank2^{P2/P2}$ N>CKIIα	$Ank2^{P2/P2}$ N>CK2α_KD	1.14E-08	2.8E-05

Genotypes		1-2 boutons on m 6/7, p	3-7 boutons on m 6/7, p	≥8 boutons on M 6/7, p
$Ank2^{P2/P2}$	$Ank2^{P2/P2}$ N>CK2α	0.348161	0.159462	0.388867
$Ank2^{P2/P2}$	$Ank2^{P2/P2}$N>CK2α_KD	0.739442	0.000872	0.464997
$Ank2^{P2/P2}$ N>CK2α	$Ank2^{P2/P2}$N>CK2α_KD	0.140445	5.23E-06	0.080816

Genotype	Retraction frequency (%)				
	M 6/7	p	M 1/9, 2/10	p	n
Wild type	2.1±1.4		1.0±0.7		12

$CK2\alpha^{P1/+}$	2.1±1.4	1.0	1.0±0.7	1.0	12
$Ank2^{P2/+}$	3.1±1.7	0.633323	1.6±0.8	0.633323	12
$CK2\alpha^{P1/+}Ank2^{P2/+}$	3.1±1.7	0.633323	1.6±0.8	0.633323	12
$CK2\alpha^{P1/P1}$	52.4±5.7	1.84E-08	58.2±3.2	1.82E-13	21
$CK2\alpha^{P1/P1}Ank2^{P2/+}$	77.3±5.0	5.89E-09	82.4±3.1	4.17E-11	11
$Ank2^{P2/P2}$	60.8±4.5	6.43E-10	65.1±4.2	4.24E-09	15
$Ank2^{P2/P2}CK2\alpha^{P1/+}$	87.5±4.5	1.47E-11	91.1±3.0	5.54E-14	14

Genotypes		M 6/7, p	M 1/9, 2/10, p
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}Ank2^{P2/+}$	0.002686	1.31E-05
$Ank2^{P2/P2}$	$Ank2^{P2/P2}CK2\alpha^{P1/+}$	0.000295	6.8E-05

Genotypes		1-2 boutons on m 6/7, p	3-7 boutons on m 6/7, p	≥8 boutons on M 6/7, p
$CK2\alpha^{P1/P1}$	$CK2\alpha^{P1/P1}Ank2^{P2/+}$	0.114004	0.103222	0.484286
$Ank2^{P2/P2}$	$Ank2^{P2/P2}CK2\alpha^{P1/+}$	0.289262	0.049848	0.143544

Materials and Methods

Fly Stocks

Flies were maintained at room temperature on standard food. Crosses and RNAi experiments were performed at 25 °C unless otherwise indicated. The following fly strains were used in this study: w^{1118} (wild type), da-Gal4, UAS-mCD8-GFP, P(hsFLP)86E, P(hsFLP)1, P(neoFRT)19A, CK2α^{Tik} (#24512), CK2α^{TikR} (#24511) (Bloomington Drosophila Stock Center, USA); dnMts (Hannus et al., 2002); PP2A-29B^{GE16781} (GenExel, South Korea); BG57-Gal4, sca-Gal4 (Budnik et al., 1996); UAS-dic2 (Dietzl et al., 2007); elav^{C155}-Gal4 (Luo et al., 1994); mef2-Gal4 (Ranganayakulu et al., 1996); CK2β^{MbuP1}, and CK2$\beta^{Mbu26-2}$ (Jauch et al., 2002). Most RNA interference stocks were ordered from the Vienna Drosophila RNAi Center (Dietzl et al., 2007) (listed in Supplement table 1). Two RNAi stocks were ordered from the Bloomington Drosophila Stock Center: y1v1; P(TRIP.JF01792)attp2 (#25786, CKIα RNAi) and y1v1; P(TRIP.JF03316)attp2 (#29384, PP2A-29B RNAi). Two stocks containing a p-element insertion in CK2α gene were obtained from the Kyoto Stock Center: CK2α^{P1} (#141869): yw; PBac(SAstopDsRed)LL05896 P(FRT(whs))2A P(neoFRT)82B P(Car20y)96E/Tm6b, Tb1 and CK2α^{P2} (#141994): yw; PBac(SAstopDsRed)LL07221 P(FRT(whs))2A P(neoFRT)82B P(Car20y)96E/Tm6b, Tb1.

Generation of CK2α and CK2β Constructs and Transgenes

The full length CK2α and CK2β open reading frames were amplified by PCR from LD27706 and RE31047 plasmids respectively (both obtained from the Drosophila Genomic Research Center, USA). Full length constructs were subcloned into the pENTR vector by TOPO cloning (Invitrogen). For in vivo expression, cDNAs were subcloned into pUAST plasmids by gateway cloning using standard protocol (Invitrogen). Point mutations were introduced into pENTR clones using the QuickChange II site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing (FMI Sequencing Facility). The constructs were sent to the Genetic Services Inc., USA, to produce UAS-CK2α, UAS-CK2β, UAS-CK2α_K66M (kinase-dead), UAS-CK2α_M161K, UAS-CK2α_L39A, UAS-CK2α_F52A, UAS-CK2α_L39A F52A, UAS-CK2α_E165A, and UAS-CK2α_E165D flies. The following transgenic fly lines

were generated in our lab using standard embryo microinjection procedures: UAS-CK2α_E165A R167A K168A, UAS-GFP-CK2α, and UAS-GFP-CK2β. All transgenic stocks were confirmed by sequencing (FMI Sequencing Facility). Primers used in this study are listed in Supplemental Experimental Procedures.

Immunohistochemistry

Wandering third instar larvae were dissected in standard dissecting saline and fixed with 100% Bouin's solution for 2 minutes (Sigma-Aldrich). Excess fixative was removed by extensive washing in PBS+0.1% Triton-X (PBT). Larvae were incubated in PBT with primary antibodies overnight (4 °C), washed in PBT, and incubated in PBT with fluorescent-conjugated secondary antibodies for 2 hours at room temperature. The larvae were mounted in Prolong Gold (Invitrogen) for imaging analysis. Primary antibodies were used at the following dilutions: anti-Bruchpilot (nc82) 1:250, anti-Synapsin (3c11) 1:50, anti-Nrg180 (BP104) 1:400 (all provided by Developmental Studies Hybridoma Bank, Iowa); rabbit anti-Dlg 1: 30 000 (Budnik et al., 1996), rabbit anti-DGluRIII 1: 2500 (Marrus et al., 2004); rabbit anti-DvGlut 1:40 000, rat anti-CD8 1:1000 (Caltag Laboratories); mouse anti-GFP (3E6) 1:1000, rabbit anti-GFP (A6455) 1:2000 (Invitrogen). Monoclonal rat anti-Ank2L 1:40 was produced against a protein fragment containing 3134-3728 aa. Antibodies were generated at the David's Biotechnology (Regensburg, Germany).

All Alexa conjugated secondary antibodies (Invitrogen) were used at 1:1000. Directly conjugated anti-HRP (Cy-dyes) were used at the following dilutions: anti-HRP Cy3 1:1000, anti-HRP Cy5 1:400 (Jackson Immunoresearch Laboratories). Images were captured using a confocal Leica SPE microscope. Individual nerves/synapses were optically sectioned at 0.25 μm using a z-stack. All genotypes for the same experiment were imaged at the same gain and set. All images were taken at room temperature using Immersol imaging oil (Carl Zeiss, Inc). Image analysis and processing, specifically cropping and adjustment levels, was done in Imaris (Bitplane) and Adobe Photoshop.

CK2α and CK2β antibody production

Rabbit anti-sera against Drosophila CK2α and CK2β were raised using bacterially-expressed His-tagged full length proteins as immunogens (primers are listed in supplemental

experimental procedures). His SpinTrap columns were used to purify His-CK2α and His-CK2β according to the manufacturer's procedures (GE Healthcare, UK). Since both were insoluble proteins, urea buffers for denaturing conditions were used to bind and elute the proteins. Monoclonal rabbit CK2α and CK2β antibodies were generated at the David's Biotechnology (Regensburg, Germany).

Quantification of phenotypes

Synaptic retractions were quantified using presynaptic Brp and postsynaptic DGluRIII staining and counting the number of glutamate receptor puncta lacking the opposing Brp marker (synaptic footprints) (segments A3-A6, m1/9, 2/10, m 6/7, and m 4). N indicates the number of independent animals per quantification.

Presynaptic Nrg, Ank2L/XL, and DvGlut levels were measured in the most distal, middle, and proximal boutons of a given NMJ branch using Imaris (Bitplane) (segment A4, m 4). Proximal bouton is defined as the bouton situated closest toward the site where a given NMJ branches out. Distal bouton is defined as the bouton situated at the terminal of a given NMJ, away from the point of branching out. DvGlut staining was used to quantify the average DvGlut cluster number (segments A2-A6, m 4). Satellite bouton numbers were quantified using HRP staining (segments A4 and A5, m 4). Boutons were counted as ''satellite'' if they budded off from one central parent bouton on the main branch to form smaller boutons. The axonal trafficking defects were quantified using Brp, DvGlut and HRP staining. The Fiji Software was used to measure Brp/DvGlut fluorescence intensity in axons. Bouton number was quantified (segments A2-A6, m 4) using Synapsin/Dlg staining. N indicates the number of analyzed NMJs.

Western Blot and Immunoprecipitation

Larval brains were dissected in dissection buffer with added protease inhibitors (Roche), transferred into 2x sample buffer (Invitrogen), and boiled for 10 minutes at 95 °C. Samples were run on 14% NuPage gels (Invitrogen) according to standard procedures. Gels were transferred to PVDF membrane for 2 hours at 30V, and then membranes were probed with primary antibodies overnight at 4 °C. The primary antibodies were used at the following concentrations: rabbit anti-CK2α 1:400, rabbit anti-CK2β 1:400, mouse anti-CK2β (6D5) 1:200 (Calbiochem), and mouse anti-Tubulin (E7) 1:1000 (Developmental Studies Hybridoma Bank). Secondary HRP-

conjugated goat anti-mouse or goat anti-rabbit antibodies were used at 1:10 000 (Jackson Immunoresearch) for 2 h at room temperature. Membranes were incubated with ECL substrate (SuperSignal West Pico Kit, Thermo scientific) and developed on film (Fujifilm).

For immunoprecipitation experiments, Drosophila S2 cells were co-transfected with act5C-Gal4, UAS-HA-CK2β, and either one of the following plasmids: UAS-GFP-CK2α_wt, UAS-GFP-CK2α_KD, UAS-GFP-empty, UAS-GFP-CK2α_L39A, UAS-GFP-CK2α_F52A, or UAS-GFP-CK2α_L39A F52A. Fugene (Roche) was used following the manufacturer's instructions. The S2 cells were lysed using the TNT lysis buffer, transferred to lo-bind Eppendorf tubes, incubated on ice for 30 minutes, and centrifuged for 10 minutes (top-speed, 4C). An aliquot was taken to analyze the input, and GFP-TrapA beads (Chromotek) were used to pull down CK2α/β according to the manufacturer's instructions. IP`s were analyzed using mouse anti-HA (12CA5) 1:200, rabbit anti-GFP (Molecular Probes) 1:500, and mouse anti-Tubulin 1:1000. Eight independent IP experiments were performed.

MARCM analysis

The *CK2β* null allele $CK2\beta^{Mbu26\text{-}2}$ (Jauch et al., 2002) was recombined with the P(neoFRT)19A chromosome. The $CK2\beta^{Mbu26\text{-}2}$ P(neoFRT)19A stock was crossed to P(hsFLP)1, P(neoFRT)19A, tubGal80; *ok371*-Gal4, UAS-CD8-GFP; MKRS, P(hsFLP)86E. Parents were removed from the vile 2 hours after egg laying and embryos were aged for additional 3 hours. The vile with embryos was taken to the water bath at 37.5 °C for 1 hour and to an 18 °C incubator overnight.

***In Vitro* Phosphorylation Analysis**

In vitro phosphorylation was performed by incubating 2.5 μg of Ank2-R1 construct (Ank2 1682-2089 aa fragment) (Pielage et al., 2008) with 250 units of CK2 and 2 pmol (γ-^{32}P)ATP (3000 Ci/mmol) for 30 minutes at 30 C in 50 μl of 20 mM Tris (pH=7.5), 50 mM KCl, 10 mM MgCl2, 0.1 mM ATP (New England BioLabs). When indicated, 30 μM TBB was added to the reaction to inhibit CK2. The phosphorylation assays were repeated in the same manner without (γ-^{32}P) ATP. The constructs were precipitated according to standard procedures (TCA precipitation) and sent to mass spec analysis (FMI Protein Analysis Facility).

Statistical analysis

All statistical analyses were performed using Microsoft Office Excel and Student's two-tailed T-test. Significant differences between compared groups were noted by asterisks (* ≤0.05, **≤0.01, ***≤0.001)

Supplementary Experimental Procedures

Table of primers

Primer	*DNA-sequence*	
CK2α-ENTR-N-term	5`CACCATGACACTTCCTAGTG	
CK2α -ENTR-C-term	5`TTATTGCTGATTATTGGGAT	
CK2β-ENTR-N-term	5`CACCATGAGCAGCTCCGAGGAAG	
CK2β-ENTR-C-term	5`TTAGTTTTTCGCTCGTAGTGG	
CK2α K66M kinase-dead	5`GGAAAAGTGCGTTGTTATGATTCTGAAACCTG	
CK2α L39A	5`GCAATCAAGACGATTATCAGGCGGTCCGTAAATTAGGCCG	
CK2α F52A	5`GTATTCTGAGGTCGCCGAGGCCATTAATATTACGACCACGG	
CK2α M161K	5`GCATCGTGATGTAAAGCCCCACAATGTTAAGATAGATCACG	
CK2α E165D	5`GTTATGATAGATCACGATAATCGAAAATTGCGCCTTATAG	
CK2α E165A	5`GTTATGATAGATCACGCAAATCGAAAATTGCGCCTTATAG	
CK2α E165A R167A K168A	5`GATAGATCACGCAAATGCAGCATTGCGCCTTATAG	
Check and seq primer	*Forward primer*	*Reverse primer*
pENTR_CK2αwt, K66M, L39A, F52A, M161K, E165A R167A K168A(sequencing of complete ORF)	5`CACCATGACACTTCCTAGTG 5`GTTCGTGTGGCTTCGAGATAC 5`GGAAAAGTGCGTTGTTATGATTCTGAAACCTG	5` CCCATGCTGTGGCAGTAG 5`GTGACGCTGTAGAATGTC 5`TTGCTGATTATTGGGATTC
pENTR_CK2βwt (sequencing of complete ORF)	5`CACCATGAGCAGCTCCGAGGAAG 5`CGCGGCATCGCTCAAATG	5`TTAGTTTTTCGCTCGTAGTGG 5`CTCGCAGAAGAACTCATTGC 5`AACCATTGCCTCGCCGGG
pENTR _CK2α/βwt (check)	5`GTAAAACGACGGCCAG	5`CAGGAAACAGCTATGAC
pENTR/pUAST CK2α/βwt (check)	5`TATAAATAGAGGCGCTTCGT	5`AGATCCCGCGGCTCTAGTTC
10xpUAST_CK2α/β_EGFP (check)	5`ACAACCACTACCTGAGCACCCA	5` AGATCCCGCGGCTCTAGTTC
10xpUAST_CK2α/β_HA (check)	5`ATGGATCtCCACcGCGGTGGAGGC	5` AGATCCCGCGGCTCTAGTTC
pENTR/pDEST17 His-tag-CK2α/βwt (check)	5`TAATACGACTCACTATAGGG	5`GCTAGTTATTGCTCAGCGG

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ADDITIONAL DATA

I identified CK2 as an essential molecule for Drosophila NMJ stability and the adaptor molecule Ank2-L as its potential substrate. If CK2 signaling functions locally at the synapse and modifies Ank2-L, one would expect CK2 to be present at the NMJ. Therefore, I addressed the localization of CK2α, CK2β, and Ank2-L at the NMJ. Because CK2α and CK2β antibodies did not work *in situ*, I generated GFP-tagged CK2α and CK2β and expressed these constructs in the motorneurons to assess their distribution. Both CK2α and CK2β were distributed within bouton and inter-bouton regions and partially co-localized with Ank2-L within the presynaptic nerve terminal (Figure 9). In addition, kinase-dead CK2α ($CK2\alpha^{KD}$) showed the same distribution as wild type CK2α and also partially co-localized with Ank2-L (Figure 9C). Interestingly, I also observed that GFP-CK2β appeared in a slightly punctuate pattern in comparison to GFP-CK2α or GFP-$CK2\alpha^{KD}$ (Figure 9B, 10B, E). These data are consistent with my previous results. If, in fact, Ank2-L is an *in vivo* substrate of CK2 then I expect at least partial co-localization of both molecules within the NMJ. Therefore, CK2 is able to physically interact with and potentially phosphorylate Ank2-L.

In addition to controlling synapse stabilization, CK2 is important for regulation of additional aspects of synapse development, such as organization of synaptic domains (this study). Thus, I also assessed the CK2 distribution with respect to several presynaptic markers, such as the active zone marker Bruchpilot (Brp) and synaptic vesicle domain marker Synapsin (Syn). Both wild type and kinase-dead GFP-CK2α partially overlapped with Brp and Syn (Figure 10A, C, D, F). CK2β, on the other hand, showed almost no co-localization with Brp (Figure 10B) and had a minor overlap with Syn (Figure 10E). This could reflect the three-dimensional arrangement of CK2 holoenzyme within boutons. Collectively, these data show that CK2 can localize to the presynaptic nerve terminal and physically interact with a variety of synaptic molecules consistent with its local regulatory role at the NMJ.

Mutations in CK2α and knock down of CK2β using RNAi also caused an active zone protein Brp to accumulate in axons of larval nerves (Figures 11-12, Supplement table 3). Similar results were observed for synaptic vesicle domain protein DvGlut (Figure 12), indicating that it is a general defect and not specific to the transport of Brp protein. However, I observed the

differential localization of Brp and DvGlut aggregates in different axonal regions. Brp accumulated both in the anterior and posterior axonal regions (Figure 11), whereas DvGlut accumulated almost exclusively in the most posterior parts of motorneurons (Figure 12). The extent of trafficking defects was identical in CK2α mutant and CK2β RNAi animals. Both genotypes showed more than a 2-fold increase of average Brp intensity in the anterior axonal regions and more than a 3-fold increase of both Brp and DvGlut intensities in the posterior axonal regions (Figure 11F-G, 12F-G). This is consistent with my previous results implying that CK2 functions as a holoenzyme in Drosophila. Importantly, the axonal Brp and DvGlut accumulation phenotype was rescued by presynaptically expressing wild type, but not kinase-dead, CK2α in CK2α mutants (Figure 11D, E, F, G, 12D, E, F, G). This suggests that kinase function of CK2 is indispensable for preventing defects in axonal transport.

The reason for abnormal accumulations of proteins in the axon in CK2α mutant and CK2β RNAi animals could be due to a general impairment of the axonal transport machinery due to the loss of CK2 function. Additionally, Brp and DvGlut could appear in the axons as a secondary consequence of retrograde transport from the NMJs actively undergoing synapse disassembly processes. In support of this model, I observed major axonal Brp/DvGlut aggregations in close proximity to ongoing retractions (data not shown). This suggests that Brp and DvGlut are indeed transported away from the NMJ during synapse disassembly and might end up accumulating in the motorneuron axons. In addition, higher levels of Brp/DvGlut in the posterior in comparison to anterior axonal regions (Figure 11F, G, 12F, G) could reflect a higher retraction rate in the posterior in comparison to anterior NMJ segments (data not shown). Although I did not observe any DvGlut aggregates in most anterior axonal segments, where retractions are rare, Brp aggregates were still present there. Therefore, I prefer the interpretation that both general axonal transport defects and synapse disassembly could account for axonal Brp/DvGlut accumulations.

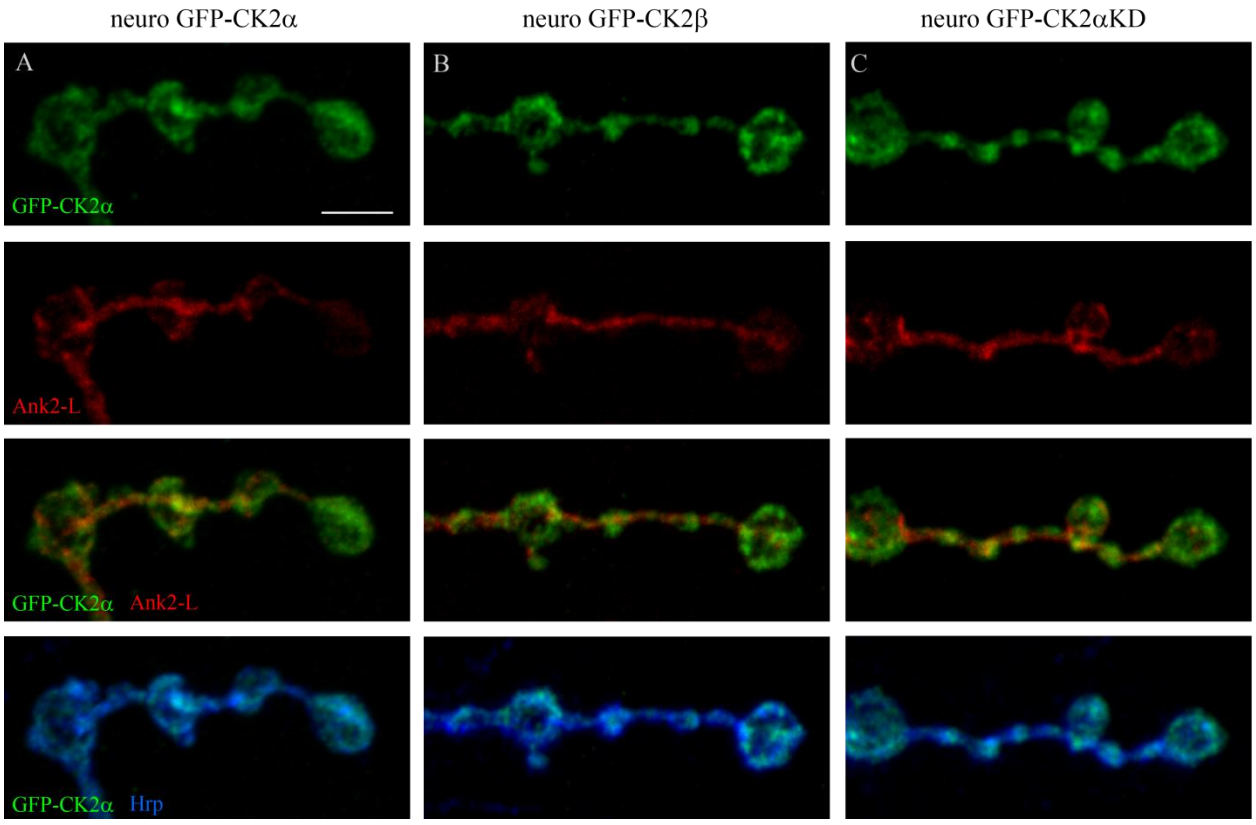


Figure 9 – CK2 partially overlaps with Ankyrin2-L

(A-C) Muscle 4 NMJs stained with an antibody specific to the long isoform of the adaptor molecule Ankyrin-2 (Ank2-L, red), the green fluorescent protein (GFP, green), and the presynaptic motorneuron membrane marker Hrp (blue). Scale bar: 5 μm. Neuronally expressed GFP-tagged wild type and kinase-dead CK2α and CK2β were localized to synaptic bouton and inter-bouton regions and partially overlapped with Ank2-L.

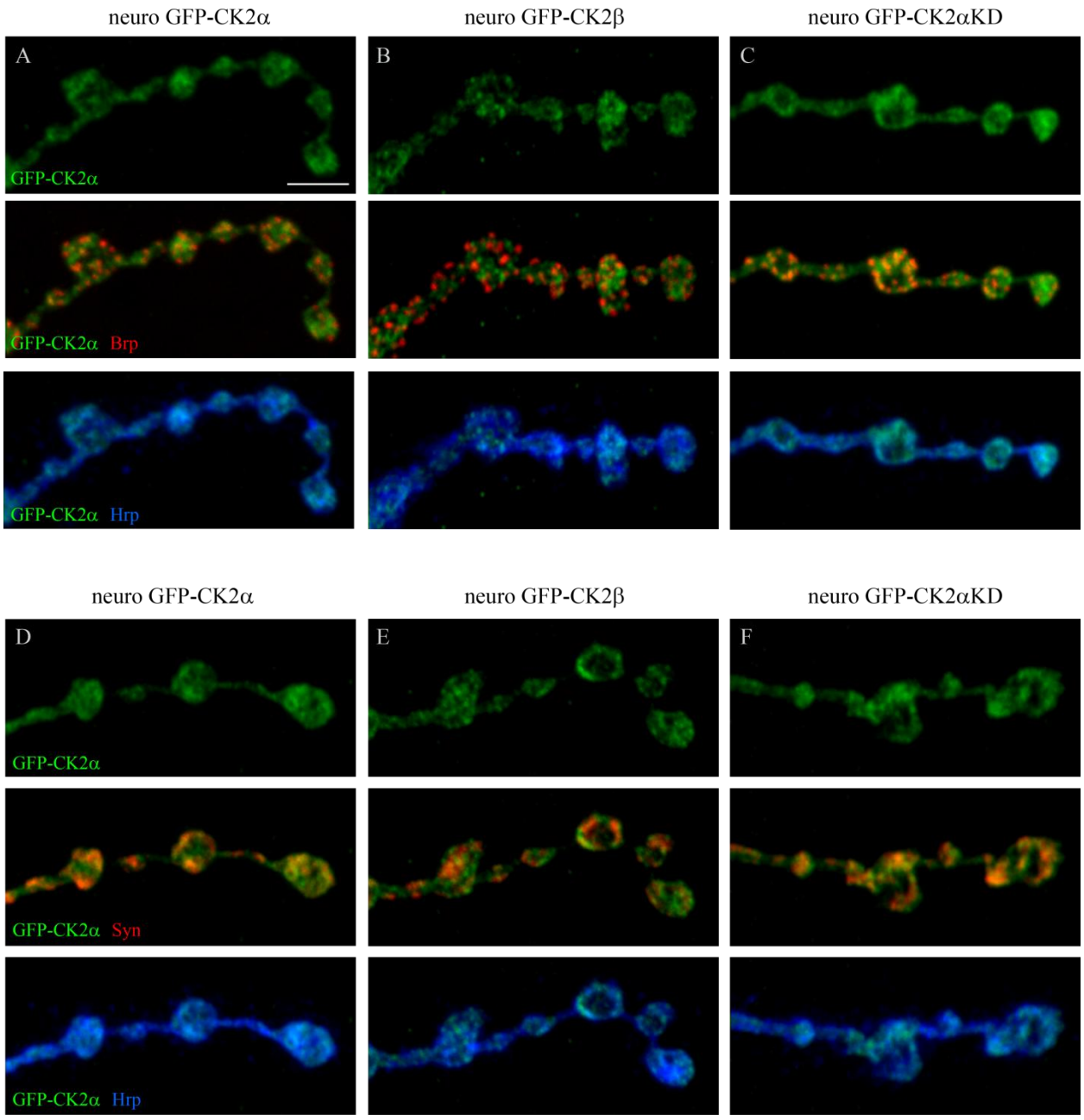
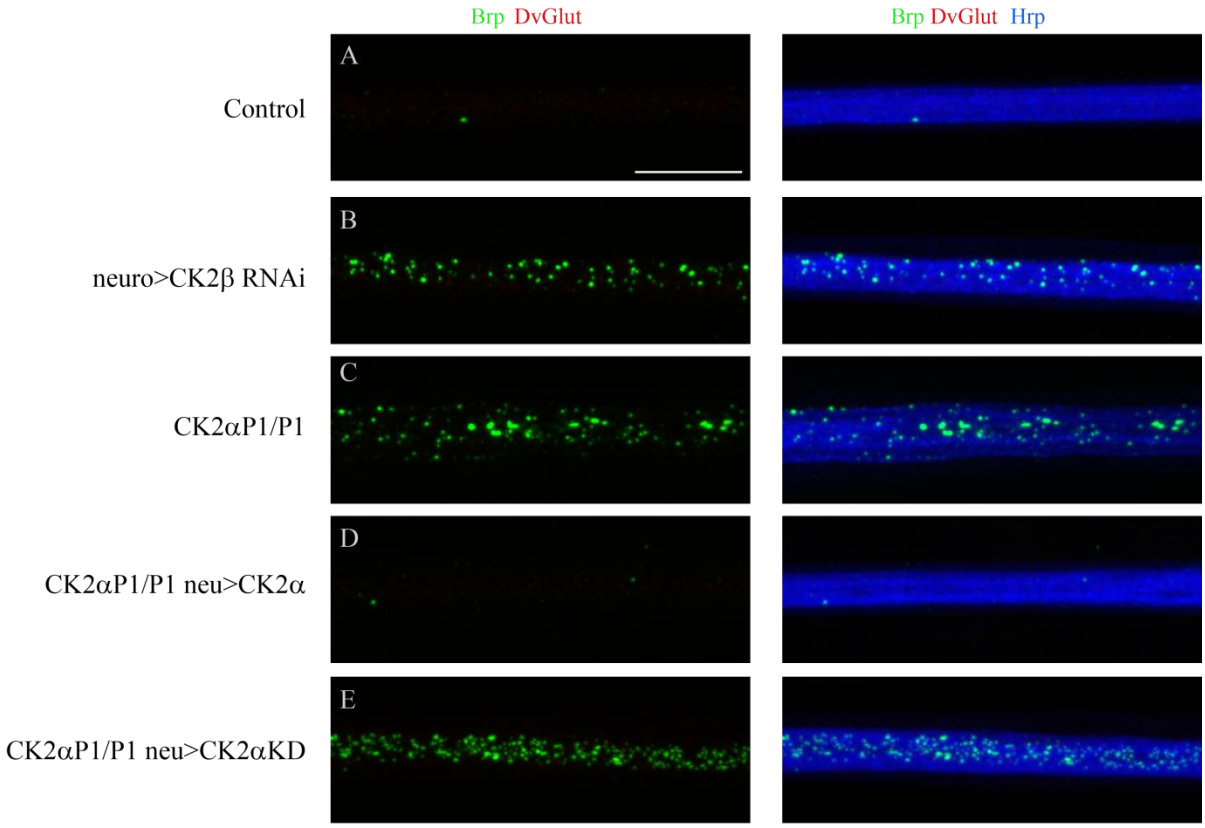
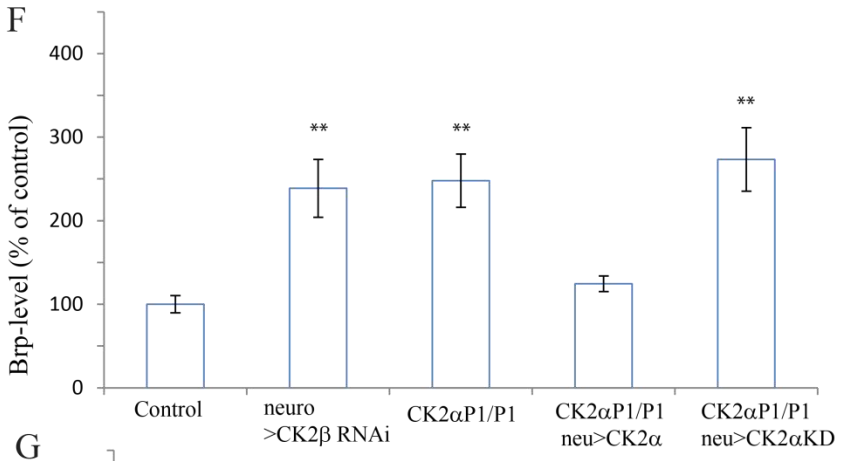


Figure 10 – CK2 can localize to the presynaptic nerve terminal and partially overlap with presynaptic active zones and synaptic vesicle domains

(A-C) NMJs on muscle 4 stained for the green fluorescent protein (GFP, green), the presynaptic active zone marker Brp (red), and the presynaptic motorneuron membrane marker Hrp (blue). Scale bar: 5 μm. **(A, C)** Neuronally expressed GFP-tagged wild type and kinase-dead CK2α could efficiently localize to the presynaptic nerve terminal. As indicated by the GFP and Hrp staining, CK2α was localized to synaptic bouton and inter-bouton regions and partially overlapped with synaptic active zones marked by Brp. **(B)** Neuronally expressed GFP-tagged CK2β could efficiently localize to the presynaptic nerve terminal in a slightly punctuate pattern. As indicated by the GFP and Hrp staining, CK2β was localized to

synaptic bouton and inter-bouton regions. It did not co-localize with synaptic active zones marked by Brp. **(D-F)** NMJs on muscle 4 stained for the green fluorescent protein (GFP, green), the presynaptic vesicle marker Syn (red), and the presynaptic motorneuron membrane marker Hrp (blue). Scale bar: 5 μm. **(D, F)** Neuronally expressed GFP-tagged wild type and kinase-dead CK2α partially overlapped with synaptic vesicles marked by Syn. **(B)** Neuronally expressed GFP-tagged CK2β showed almost no co-localization with synaptic vesicles (Syn).


Brp DvGlut
Brp DvGlut Hrp
A
Control
B
neuro>CK2β RNAi
C
CK2αP1/P1
D
CK2αP1/P1 neu>CK2α
E
CK2αP1/P1 neu>CK2αKD


F
Brp-level (% of control)
400
300
200
100
0
**
**
**
Control
neuro >CK2β RNAi
CK2αP1/P1
CK2αP1/P1 neu>CK2α
CK2αP1/P1 neu>CK2αKD

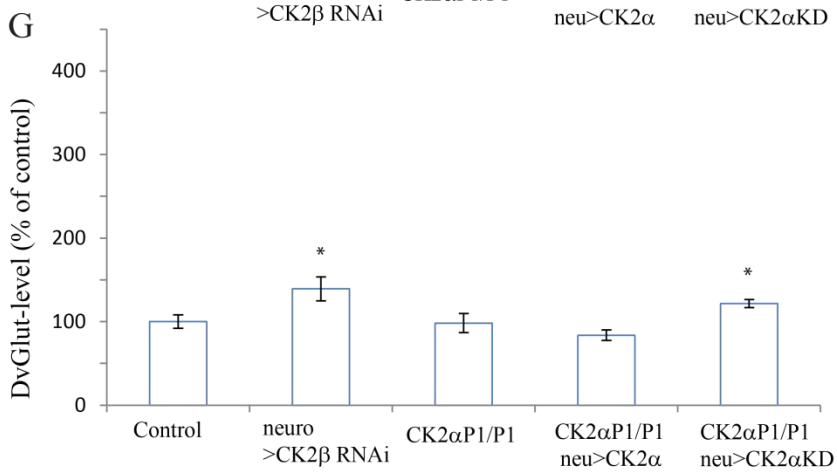
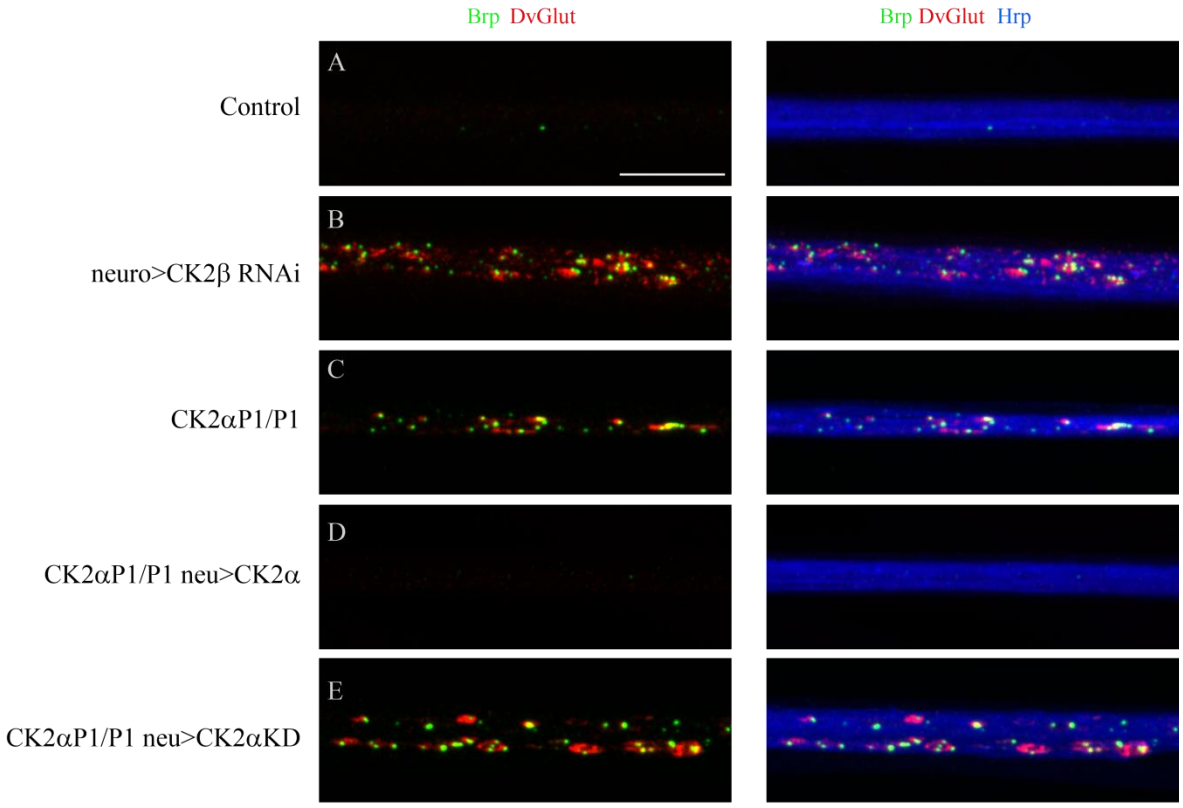
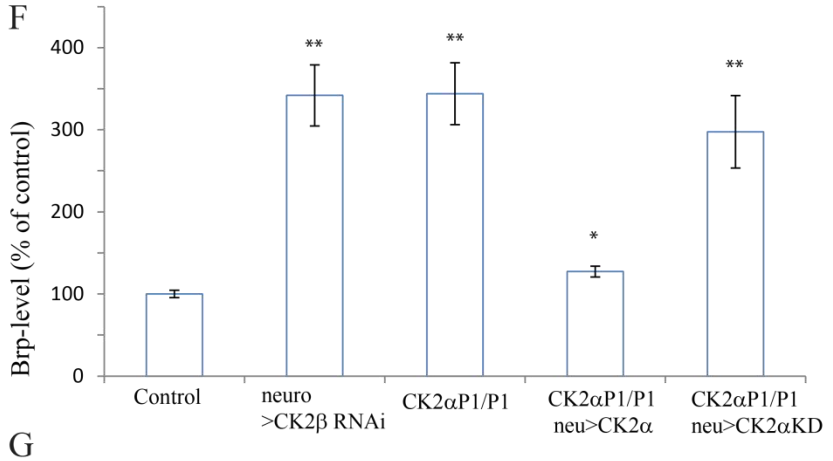

G
DvGlut-level (% of control)
400
300
200
100
0
*
*
Control
neuro >CK2β RNAi
CK2αP1/P1
CK2αP1/P1 neu>CK2α
CK2αP1/P1 neu>CK2αKD

Figure 11 – CK2 is required for normal Brp trafficking in the anterior axonal region

(A-E) Anterior motorneuron axonal regions stained for the presynaptic active zone marker Brp (green), presynaptic vesicle domain marker DvGlut (red), and the presynaptic motorneuron membrane marker Hrp (blue). Scale bar: 10 μm. **(A)** In wild type motorneuron axons very rarely contained Brp puncta and never showed DvGlut staining. The rarely observed Brp puncta were small and spherical. **(B-C)** Animals in which CK2β RNAi was presynaptically expressed (B) and CK2α mutants (C) had pronounced Brp accumulations in the anterior axonal region. Large aggregates of multiple Brp puncta were often observed. We did not observe any DvGlut trafficking defects. **(D-E)** Neuronal expression of wild type CK2α (D) but not kinase-dead CK2α (E) in CK2α mutant animals rescued the Brp trafficking defect. **(F-G)** Quantification of Brp (F) and DvGlut (G) levels in the anterior axonal regions. The values are normalized to wild type control. Significant differences between compared groups are noted by asterisks (* ≤0.05, **≤0.01, n=5-8 axons). **(F)** Loss of CK2β or CK2α caused a significant more than 2-fold increase in Brp levels in the anterior axonal region. Expressing wild type but not kinase-dead CK2α in CK2α mutants was sufficient to rescue the Brp trafficking phenotype. **(G)** There was a very small increase in DvGlut levels in CKβ RNAi and CK2α mutant animals rescued with kinase-dead CK2α. Overall, the DvGlut trafficking defects were not observed in all five genotypes (A-E) and the increase in most likely due to the differences in background.


Brp DvGlut
Brp DvGlut Hrp
A
Control
B
neuro>CK2β RNAi
C
CK2αP1/P1
D
CK2αP1/P1 neu>CK2α
E
CK2αP1/P1 neu>CK2αKD


F
Brp-level (% of control)
0
100
200
300
400
**
**
*
**
Control
neuro >CK2β RNAi
CK2αP1/P1
CK2αP1/P1 neu>CK2α
CK2αP1/P1 neu>CK2αKD

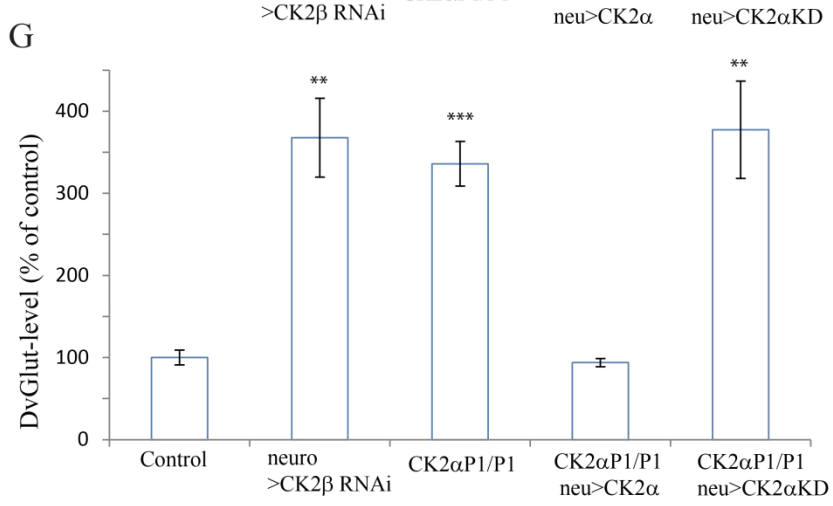

G
DvGlut-level (% of control)
0
100
200
300
400
**
***
**
Control
neuro >CK2β RNAi
CK2αP1/P1
CK2αP1/P1 neu>CK2α
CK2αP1/P1 neu>CK2αKD

Figure 12 – CK2 is required for normal Brp and DvGlut trafficking in the posterior axonal region

(A-E) Posterior motorneuron axonal regions stained for the presynaptic active zone marker Brp (green), presynaptic vesicle domain marker DvGlut (red), and the presynaptic motorneuron membrane marker Hrp (blue). Scale bar: 10 μm. **(A)** In wild type motorneuron axons very rarely contained Brp puncta and never showed DvGlut staining. **(B-C)** Animals in which CK2β RNAi was presynaptically expressed (B) and CK2α mutants (C) had pronounced Brp and DvGlut accumulations in the posterior axonal region. Large aggregates of Brp and DvGlut puncta were often observed. Brp and DvGlut puncta often co-localized **(D-E)** Neuronal expression of wild type CK2α (D) but not kinase-dead CK2α (E) in CK2α mutant animals rescued the Brp and DvGlut trafficking defect. **(F-G)** Quantification of Brp (F) and DvGlut (G) levels in the posterior axonal regions. The values are normalized to wild type control. Significant differences between compared groups are noted by asterisks (* ≤0.05, **≤0.01, **≤0.001, n=5-8 axons). Loss of CK2β or CK2α caused a significant more than 3-fold increase in Brp (F) and DvGlut (G) levels in the posterior axonal region. Expressing wild type but not kinase-dead CK2α in CK2α mutants was sufficient to significantly rescue both Brp (F) and DvGlut (G) trafficking phenotypes.

Supplemental Table 3

Brp/DvGlut trafficking defects

Genotype	Brp, % Anterior	P	DvGlut, % Anterior	P	N
Wild type	100±10.3		100±8.0		6
neu>CK2β RNAi	238.7±34.6	0.004966	139.2±14.3	0.035285	8
$CK2\alpha^{P1/P1}$	247.9±32.0	0.003148	98.2±11.4	0.8988	7
$CK2\alpha^{P1/P1}$ neu>CK2α	124.5±9.3	0.105225	83.6±6.3	0.137255	8
$CK2\alpha^{P1/P1}$ neu>CK2α_KD	273.4±38.0	0.006937	121.6±4.7	0.047285	5

Genotype	Brp, % Posterior	P	DvGlut, % Posterior	P	N
Wild type	100±4.4		100±9.1		5
neu>CK2β RNAi	341.9±37.1	0.002943	367.6±48.1	0.005438	5
$CK2\alpha^{P1/P1}$	343.9±37.8	0.003049	336.0±27.4	0.000444	5
$CK2\alpha^{P1/P1}$ neu>CK2α	127.3±6.6	0.01092	93.8±5.0	0.573351	5
$CK2\alpha^{P1/P1}$ neu>CK2α_KD	297.5±44.1	0.004313	377.4±59.2	0.003582	7

EXTENDED DISCUSSION

Kinases and Phosphatases Identified in the RNAi Screen

The reversible phosphorylation of proteins is a major mechanism for regulating many cellular functions such as cell division, proliferation, apoptosis, metabolism, signal transduction, and memory. In addition, protein phosphorylation plays an important role in regulating many aspects of synaptic development and function (Soderling and Derkach, 2000; Winder and Sweatt, 2001; Wu et al., 2010). In the RNAi screen, I identified 7 kinases and 4 phosphatases which are specifically important for synapse stabilization: CK2, Casein kinase Iα (CKIα), Minibrain (Mnb), MAST, 1-phosphatidylinositol 4 kinase (PI4KIIIα), Insulin-receptor kinase (inR), Cyclin-dependent kinase 2 (Cdc2/Cdk1), Protein phosphatase 2A 29B (PP2A-29B), CG9784, Protein phosphatase 4 19C (PP4-19C), and Multiple inositol polyphosphates phosphatase 2 (Mipp2). To my knowledge, none of these molecules were previously implicated in synapse stability. The identified kinases and phosphatases are involved in various signaling pathways.

Lipid/Inositol Signaling

Inositol kinase PI4KIIIα and two inositol phosphatases CG9784 and Mipp2 detected in the RNAi screen are involved in lipid/inositol signaling pathways. PI4KIIIα has only been recently described, and virtually nothing is known about CG9784 and Mipp2 in mammals or fly.

It is well established that the main function of inositol kinases and phosphatases is to regulate the phosphorylation of various phosphoinositides – lipids that are present in the cytoplasmic leaflet of a cell's plasma and internal membranes. Phosphoinositides, in turn, play fundamental roles in various neuronal processes, e.g. synaptogenesis, neurotransmitter release, synaptic vesicle recycling, ion channel activity, regulation of actin dynamics in the presynaptic compartment, synaptic polarity, and growth (Cremona and De Camilli, 2001; Cremona et al., 1999; Khuong et al., 2010; Kimata et al., 2012; Martin-Pena et al., 2006). A wide variety of molecules can attach to phosphoinositides, such as protein kinases, phospholipases, ion channel proteins, scaffold proteins, cytoskeletal proteins, and regulators of membrane trafficking (Di Paolo and De Camilli, 2006; Sasaki et al., 2007; Vicinanza et al., 2008). Thus, the distinct isozyme-specific properties of phosphoinosities can be generated via regulating phosphorylation

of different hydroxyl residues of the inositol ring, and kinases and phosphatases play a fundamental role in this process.

Lipid signaling plays a crucial role in the development of the nervous system (Lo Vasco 2012). For example, PI4KIIIα mediates the formation of phosphatidylinositol-4-monophosphate (PI(4)P), a precursor of $PI(4,5)P_2$. Although $PI(4,5)P_2$ signaling is involved in various cellular processes, a specific synaptic role for PI4KIIIα was only recently identified at the Drosophila NMJ by Khuong et al (Khuong et al., 2010). PI(4,5)P2 levels are important for autonomous localization and activation of a presynaptic actin-binding Wiscott-Aldrich syndrome protein (WSP) (Khuong et al., 2010). By increasing $PI(4,5)P_2$ levels, PI4KIIIα negatively regulates actin-dependent synapse growth at the NMJ via WSP-mediated signaling pathway, which is independent of synaptic bone morphogenic protein (BMP) signaling (Khuong et al., 2010). Indeed, in addition to synapse retractions, I also observed a similar synaptic overgrowth phenotype in PI4KIIIα RNAi animals described by Khuong et al. Interestingly, in contrast to PI4KIIIα, PI3K positively regulates synapse number at the Drosophila NMJ and is able to induce synaptogenesis in aged adult neurons (Martin-Pena et al., 2006). Therefore, synapse number seems to be dynamically regulated by specific kinases within the lipid signaling pathway. In addition to already identified roles in synapse growth, I identified novel roles for lipid kinases/phosphatases in synaptic stabilization.

Metabolism and Insulin Signaling

Two kinases identified in synapse stability - a receptor tyrosine kinase InR and a serine/threonine kinase Mnb – play important roles in metabolism (Hong et al., 2012). Originally, however, Mnb was implicated in regulating the number of distinct types of neuronal cells during postembryonic neurogenesis in Drosophila (Tejedor et al., 1995). Mutations in its mammalian ortholog Dual specificity tyrosine-phosphorylation-regulated kinase 1a (Dyrk1a) also led to defects in neuroblast proliferation and brain development in mice (Fotaki et al., 2002). Likewise, human patients with truncated mutations in Dyrk1a have mental retardation and microcephaly (Moller et al., 2008; van Bon et al., 2011). Thus, Mnb/Dyrk1 appears to be an essential kinase to ensure proper synaptic development and function and its novel role in synapse stabilization could contribute to the understanding of what goes wrong during disease.

Insulin receptors are abundant in the central nervous system. In addition to an obvious role in the control of food intake, insulin pathway plays an important role in regulation of synaptic plasticity, neuronal survival, axon guidance, neurite outgrowth, spine density, synapse activity, spatial memory, and cognitive function (Choi et al., 2005; Govind et al., 2001; Huang et al., 2010; Huang et al., 2003; Zhao et al., 1999). Furthermore, the InR is a structural component of synapses (Abbott et al., 1999). In Drosophila, up- or downregulation in the levels of the InR and Chico, the fly ortholog of the mammalian insulin receptor substrates (IRS), has an effect on synapse number and cell proliferation (Martin-Pena et al., 2006). Specifically, downregulating the InR levels caused fewer synaptic boutons at the Drosophila NMJ (Martin-Pena et al., 2006). In addition to synaptic retractions, I also observed fewer synaptic boutons (data not shown) in animals in which the InR was knocked down using RNAi. Therefore, in addition to regulating synaptic bouton growth/proliferation, the InR might also control synapse stability. In fact, a link between insulin receptor signaling and the structural stabilization of excitatory synaptic contacts was proposed. By regulating the connection of synaptic scaffolding proteins with the cytoskeleton, insulin signaling might play a role in stabilization of synapses (Chiu and Cline 2010). Collectively, a role of the InR signaling in synaptic function makes it a potential regulator of circuit formation, development, and stabilization.

Cell Signaling

Three serine/threonine protein kinases CK2, CKIα, Cdc2 and two serine/threonine protein phosphatases PP2A and PP4 identified in the RNAi screen are implicated in numerous cell signaling pathways. For example, CKIα, PP4, and PP2A play positive and negative roles in the Hedgehog (Hh) signaling, which is essential for normal development (Jia et al., 2009; Legent et al., 2012; Price, 2006). CK2, CKIα, and PP2A are implied in the wingless (Wnt) signaling, important for the regulation of pre- and postsynaptic differentiation (Arroyo and Hahn, 2005; Gao and Wang, 2006; Knippschild et al., 2005; Price, 2006; Wang and Jones, 2006). CK2 and PP2A are involved in the PI-3 Kinase (PI3K) signaling, which plays a pivotal role in synaptogenesis (Martin-Pena et al., 2006). A cyclin-dependent kinase Cdc2 (or Cdk1) is important for cell cycle regulation (Santamaria et al., 2007; Vermeulen et al., 2003). CK2, Cdc2, and PP2A are also implied in mitogen-activated protein kinase (MAPK)/extracellular signal-regulated (ERK) signaling pathway (Dangi and Shapiro, 2005; Junttila et al., 2008; Shi et al.,

2009; Silverstein et al., 2002; Stark et al., 2011). MAPK/ERK pathway contains a large kinase network that controls a multitude of neuronal processes (Thomas and Huganir, 2004). In summary, all identified kinases and phosphatases have the capacity to associate with constituents of several signaling transduction pathways, thereby directing their function toward specific regulatory events participating in various aspects of synapse development.

CK2 and PP2A

Interestingly, my top RNAi screen hits, CK2 and PP2A, are both implicated in the Wnt, PI3K, and MAPK/ERK signaling pathways. In addition to important roles in development, cell growth, and differentiation, all three signaling pathways are essential for such neuronal processes as synaptic assembly, transmission, and plasticity (Acebes and Morales, 2012; Budnik and Salinas, 2011; Thomas and Huganir, 2004). Both CK2 and PP2A are ubiquitous, highly conserved molecules that regulate phosphorylation of a myriad of targets. It is intriguing to think that behind the obvious randomness, CK2 and PP2A perform a number of global tasks within the cell. In most pathways, however, the specific components of the regulatory PP2A complexes and CK2 are yet to be discovered.

I originally speculated that PP2A and CK2 might regulate synapse stability through regulation of common targets in some or all of these pathways. My speculation was strengthened by the fact that the knockdown of both kinases caused similar severe retraction phenotypes and that PP2A and CK2 are known to directly interact though the invariant HENRKL sequence motif of the CK2α. CK2α binding increases PP2A activity, and CK2α-PP2A complex appears to be involved in the regulation of the MAPK signaling cascade (Heriche et al., 1997). In turn, MAPK pathway plays an important role in long-term changes at the synaptic level (Orban et al., 1999; Thomas and Huganir, 2004). Moreover, the E to D substitution in CK2α gene, comprising the Tik mutation, sits right within its HENRKL motif, crucial for PP2A binding. However, the mutational analysis, by which I abolished CK2-PP2A association, and genetic interaction experiments suggested that CK2-PP2A interaction is not required for synapse stabilization and that CK2 and PP2A most probably control synapse stability through independent signaling pathways.

Cytoskeletal Signaling

Phosphorylation dynamics are believed to play a critical role in cytoskeletal stability. Considering that retraction of the microtubule cytoskeleton appears to be one of the earliest steps during synapse elimination (Eaton et al., 2002; Pielage et al., 2008; Pielage et al., 2005), it is important to learn more about post-translational regulation of synaptic cytoskeletal, structural, and adhesive proteins. Three serine/threonine protein kinases MAST, Cdc2, and CK2 and two serine/threonine protein phosphatases PP2A and PP4 from the RNAi screen are known to bind directly to microtubules and to various microtubule-associated proteins (MAPs) (Besson et al., 2004; Fourest-Lieuvin et al., 2006; Han et al., 2009; Helps et al., 1998; Lemos et al., 2000; Lim et al., 2004; Sontag et al., 1995; Sontag and Sontag, 2006; Toyo-oka et al., 2008; Wang et al., 2012). Therefore, synaptic retractions that I observe after the RNAi knockdown of the microtubule-interacting kinases and phosphatases could be due to the disruption of synaptic microtubule cytoskeleton.

Misregulation of phosphorylation of the microtubule cytoskeleton and associated proteins could be the reason for synapse disassembly observed in CK2 mutants and/or for the altered synaptic bouton architecture in animals in which CK2α is overexpressed. Phosphorylation of tubulin by distinct kinases might affect its assembly into microtubules and its interaction with other molecules. In fact, β-tubulin from the purified brain tissue was shown to be phosphorylated by CK2 *in vitro* (Jakubowicz and Leader, 1987; Serrano et al., 1987). In addition, CK2 holoenzyme robustly stimulated microtubule assembly and bundling, and polymerized microtubules were strongly stabilized by CK2 against cold-induced depolymerization (Lim et al., 2004). Amazingly, CK2 expression can even restore the down-regulated tubulin and disrupted microtubule structures in HEK293 cells (Wang et al., 2012). Thus, CK2 plays a key role in stabilizing cellular microtubules. Surprisingly, mutational experiments demonstrated that kinase activity of CK2 is not necessary for its microtubule-stabilizing functions in cell culture (Lim et al., 2004). Therefore, it was proposed that CK2 mechanistically stabilizes microtubules through physical association with them (Lim et al., 2004). In Drosophila, CK2 enzymatic function is absolutely indispensable for synapse stabilization. I still observe a severe retraction phenotype in CK2α mutant animals rescued with the kinase-dead version of CK2α, while the wild-type CK2α perfectly rescues the phenotype. Thus, if synaptic destabilization in CK2 mutants was solely caused by CK2's direct effect on microtubules, mechanistically destabilized by its absence, both

wild type and kinase-dead CK2α should have rescued the retraction phenotype. Most probably, CK2 has other targets essential for synapse stabilization in Drosophila, which may or may not involve microtubules. Alternatively, the direct phosphorylation of microtubules by CK2 might be necessary for microtubule stability *in vivo* in Drosophila, as opposed to microtubules in cell culture.

The microtubule-associating protein MAP1B phosphorylation by CK2 is important for the microtubule assembly within neurites. CK2 is involved in phosphorylating MAP1B during neuroblastoma cell differentiation, and CK2α depletion leads to dephosphorylation of MAP1B on the corresponding phosphorylatable residues, followed by the release of MAP1B from microtubules and inhibition of neuritogenesis (Diaz-Nido et al., 1988; Ulloa et al., 1993). Collectively, the results led the researchers to speculate that MAP1B phosphorylation by CK2 may support microtubule stabilization during neurite outgrowth. The Drosophila homologue of MAP1B Futsch also regulates synaptic microtubule organization and is required for dendritic and axonal development and normal synaptic growth (Hummel et al., 2000; Roos et al., 2000). However, Futsch loss of function does not lead to synaptic retractions at the Drosophila NMJ (Roos et al., 2000). Therefore, the potential impairment of Futsch function in CK2 mutants cannot possibly explain the synaptic instability phenotype. However, the synaptic bouton morphology phenotype that I observe in CK2α mutants (bigger but less numerous boutons) could be explained by the direct impairment of Futsch function. Likewise, loss of function mutations in Futsch cause increased bouton size and reduced bouton number (Roos et al., 2000). Interestingly, animals which overexpress CK2α show the opposite bouton morphology phenotype - a dramatic increase in the number of satellite boutons and synaptic vesicle clusters but the reduction in synaptic vesicle domain size. It is also known that Futsch is necessary for elevated loop formation and increased branching (Roos et al., 2000). It is interesting to speculate that Futsch hyperphosphorylation might account for the observed phenotype in these animals. In fact, Drosophila Futsch contains 189 serines and 14 threonines potentially phosphorylated by CK2 (KinasePhos Program) (Huang et al., 2005). Therefore, phosphorylated Futsch might encourage the division of boutons by dynamically regulating microtubules within synaptic loops, and CK2 is very likely to play an essential role in this process.

Other neuronal cytoskeletal proteins including myosin heavy chain, MAP1A, Tau, and β-spectrin were also shown to be phosphorylated by CK2, however, the role of these post-translational modifications are not completely understood (Bignone et al., 2007; Clari and Moret, 1985; Diaz-Nido et al., 1988; Greenwood et al., 1994; Matsumura et al., 1983; Murakami et al., 1984). The loss of presynaptic spectrin leads to synaptic retractions at the Drosophila NMJ, so it might be a relevant CK2 target (Pielage et al., 2005). However, the two CK2 phosphorylation sites identified in vertebrate β-spectrin (Bignone et al., 2007) are not conserved in Drosophila. Although collectively these facts clearly indicate a role of CK2 in the regulation of cytoskeletal function, the specific mechanisms remain elusive.

CK2, Neuroglian and Ankyrin2

CK2 could potentially play a major role in regulating the interactions of cytoskeletal proteins such as tubulin, spectrin, and/or actin with each other and/or with cell adhesion molecules and adaptor proteins. Several such proteins have been implicated specifically in the regulation of synaptic stability in Drosophila and include the adaptor molecule Ankyrin 2 (Ank2), the actin-capping molecule Hu-li tai shao (Hts)/Adducin, and the cell adhesion molecule Neuroglian (Nrg) (Enneking 2013) (Pielage et al., 2011; Pielage et al., 2008). Possibly, CK2 ensures synapse stability via phosphorylation of key molecules within this network and thus stabilizing their interactions with the underlying cytoskeleton and/or with other important molecules.

In fact, I observe diminished levels of Nrg at the NMJs of CK2α mutants. Since there was no reduction in total Nrg protein levels in larval brains, this reduction most probably happened due to a local change in Nrg protein distribution. Given that L1/Nrg is phosphorylated by CK2 in both neuronal and non-neuronal cells during development of rat brain and is required for synapse stability at Drosophila NMJ, it might be a relevant CK2 substrate to control synapse stability (Enneking 2013) (Wong et al., 1996). I restored the Nrg levels in CK2α mutants by presynaptically overexpressing Nrg. However, increasing Nrg levels back to normal in CK2α mutant background did not rescue the synaptic destabilization. One plausible explanation is that the phosphorylation of Nrg by CK2 is absolutely crucial for synapse stability. Thus, bringing back Nrg in the background in which it cannot be phosphorylated does not allow Nrg to perform

its stabilizing functions. Alternatively, the phosphorylation of Nrg by CK2 may not be relevant for the maintenance of synapse stabilization.

Next, the adaptor protein Ank2 might be the relevant CK2 target. First, I demonstrated that CK2 phosphorylates a domain in Ank2-L isoform that can directly associate with microtubules and therefore might contribute to synapse stability. Second, Ank2 levels are significantly reduced at the NMJs of CK2α mutants suggesting that Ank2 distribution might be locally regulated by CK2. Third, overexpression of presynaptic CK2α significantly alleviates the severity of synaptic retraction phenotype caused by the partial loss of Ank2. Possibly, CK2 phosphorylation of the remaining synaptic Ank2 is enhanced, thus increasing Ank2's interaction with microtubules and/or other key synaptic proteins and making the network more stable. In contrast, overexpressing the kinase-dead version of CK2α in Ank2 mutant background causes more synaptic instability. Fifth, I demonstrated that CK2 and Ank2 genetically interact. Removing one copy of CK2α in Ank2 mutant animals and removing one copy of Ank2 in CK2α mutant animals significantly increased the frequency of synaptic retractions, implying their collaboration in the regulation of synaptic stabilization. In summary, these data suggest that Ank2 might be a key target of CK2 to regulate synapse morphology and/or stability.

It was previously demonstrated that CK2 constitutively binds to and phosphorylates chicken erythroid ankyrin and inhibits its ability to bind to spectrin *in vitro*, which is inconsistent with our results (Ghosh et al., 2002). If Drosophila Ank2 hyperphosphorylation by CK2 caused its dissociation from spectrin, I would expect to see a synaptic instability phenotype in animals overexpressing CK2α, but not in CK2 mutants. The reason for such discrepancy is not known. Since the conclusions about Ankyrin-Spectrin binding made by Ghosh et al. were based solely on the data from *in vitro* assays, they may not reflect the real situation *in vivo*. It could be that in addition to Ank2, CK2 phosphorylates other important constituents which indirectly stabilize the network through interaction with Ank2 and other components. This could explain the genetic interaction between Ank2 and CK2α and the partial relief of the synaptic retraction phenotype in Ank2 mutant animals overexpressing CK2α. Possibly, CK2 overexpression could also up-regulate certain ''stabilizing'' signaling cascades at the Drosophila NMJ. For example, it is known that enhanced MAPK-JNK-Fos signaling suppresses synapse disassembly despite the loss of presynaptic α-spectrin or Ank2 (Massaro et al., 2009). This signaling cascade is up-regulated

as a result of acute neurological stress during the disruption of the cytoskeleton (Massaro et al., 2009). It is intriguing to speculate that the significant reduction of synaptic retraction frequency in Ank2 mutants could be due to the CK2-mediated activation of yet unidentified neuroprotective signaling cascades.

Therefore, CK2 might be one of the numerous protein kinases that participate in the assembly of cytoskeleton-related multiprotein complexes to synchronize signaling pathways underlying synaptic plasticity events. Interestingly, CK2 activity and microtubule-associated protein phosphorylation have been implicated in the development of a number of neurodegenerative diseases (such as Alzheimer's), suggesting that additional studies are needed for a better understanding of molecular processes regulating microtubule dynamics during development and disease (Gong et al., 2000; Perez et al., 2011).

CK2α and CK2β Subunits Stability in Drosophila

For a long time, it was accepted in the field that CK2 exists exclusively as an obligate heterotetrameric complex *in vivo* (Pinna and Meggio, 1997). However, the traditional view was recently challenged by X-ray crystallography and by studies demonstrating that unbound α and β subunits can move independently in living cells for certain functional and regulatory reasons (Filhol et al., 2003; Niefind et al., 2001). In addition, CK2β dimers devoid of CK2α were identified in mouse brain and testicles (Guerra et al., 1999). Abnormally high levels of CK2β relative to CK2α were also observed in tumors, emphasizing the importance of understanding the dynamic role of CK2β, whether it is a part of CK2 heterotetramer or not (Vilk et al., 2001). Collectively, such findings raise the possibility that CK2 tetramers are subjects to at least transient disassembly and reassembly processes. However, there have been conflicting studies to whether the isolated α and/or β subunits are indeed present in cells *in vivo*. In fact, a number of studies suggest that α subunits can perform their functions independently of β, whereas β dimers are often degraded unless they associate with α (Canton et al., 2001; Luscher and Litchfield, 1994; Stigare et al., 1993; Zhang et al., 2002).

In Drosophila, it is not known whether α or β subunits can exist outside the CK2 complex. Mutations in two cysteine residues of CK2β responsible for the heterotetramer

assembly result in a CK2β protein unable to rescue the lethality of the CK2β loss-of-function mutant (Jauch et al., 2002). Therefore, the proper CK2α–CK2β association is absolutely essential for fly viability. The failure of CK2β cysteine mutants to substitute for the loss of endogenous CK2β could be due the fast degradation of CK2β mutated protein unable to incorporate into stable CK2 complexes. In fact, my results support this suggestion. Two P-element insertions (P1 and P2) in CK2α gene impair its transcription/translation, and therefore the protein levels of CK2α are significantly diminished. In parallel, I observe a dramatic reduction in the protein levels of CK2β in these animals, suggesting that CK2β gets degraded unless it can find and bind to CK2α. Likewise, the TikR mutation in CK2α leads to diminished CK2β levels. It is believed that TikR mutation causes a major misfolding of CK2α protein, which impairs its binding to CK2β and incorporation into the holoenzyme (Kunttas-Tatli et al., 2009). In all probability, I observe drastically diminished CK2β levels in TikR mutants because CK2β dimers get unstable without a binding partner. In contrast, the Tik mutation in CK2α does not cause CK2α misfolding or affect its levels, but it does impair its enzymatic function (Kunttas-Tatli et al., 2009). Correspondingly, the protein levels of CK2β remain normal, suggesting that the α-β interaction alone is sufficient to stabilize CK2β against degradation, even if it associates with a dysfunctional CK2α. In addition, L39AF52A mutations in CK2α not interfering with its enzymatic function but interfering with its ability to form complexes with the β-dimers (Raaf et al., 2011) also led to a major reduction of CK2β levels in CK2α null animals rescued with $CK2\alpha^{L39AF52A}$. On the other hand, I demonstrated that CK2α remains relatively stable in the absence of CK2β, as levels of CK2α are only modestly diminished in animals in which CK2β is knocked down using RNAi and not diminished at all in $CK2\beta^{P1/26-2}$ mutants. In addition, CK2α null animals rescued with $CK2\alpha^{L39AF52A}$ show no reduction in CK2α, even though its association with CK2β is largely disrupted. Therefore, my results support the statement that α subunits can exist in cells independently of β, whereas β dimers are degraded unless they associate with α. However, this does not exclude the possibility that α subunits may still briefly dissociate from β dimers in Drosophila cells *in vivo*.

ADDITIONAL MATERIALS AND METHODS

Quantification of Brp/DvGlut trafficking phenotypes

The axonal trafficking defects were quantified using Brp, DvGlut and Hrp staining. The Fiji Software was used to measure Brp/DvGlut fluorescence intensity in axons. N indicates the number of analyzed axons.

Buffers and Solutions

PBS (10X): 76 g NaCl, 9.9 g Na_2HPO_4 (sodium phosphate dibasic), and 4.1 g NaH_2PO_4 (sodium phosphate monobasic) were dissolved in 900 ml of water. The volume was brought up to 1 L using a graduated cylinder (pH=6.7). The solution was poured into bottles and autoclaved.

PBT (10X): 76.0 g NaCl, 9.9 g Na_2HPO_4 (sodium phosphate dibasic), and 4.1 g (sodium phosphate monobasic) were dissolved in 900 ml of water. Ten ml Triton X-100 were added and the volume was brought up to 1 L in a graduated cylinder (pH=7). The solution was poured into bottles and autoclaved.

Dissecting saline (1X): 4.0 g NaCl, 4.1 g $MgCl_2 \cdot 6H_2O$, 0.36g KCl, 1.2 g HEPES, 0.8 g $NaHCO_3$, 39.2 g sucrose, 40 ml EGTA (0.5M) were dissolved in water to make 1 L (pH=7.0). If needed, phosphatase (20mM NaF, 20mM sodium orthovanadate) and/or protease (1 tablet protease inhibitors, Roche, EDTA-free per 10ml) inhibitors were added right before use.

Fly food (30 L): 107 g Fadenagar and 62 g USB Agar were boiled in 17 L water. 2140 g corn meal, 480 g dry yeast, 270 g soy meal, 2140 g malt extract, 600 g treache, and ≈10 L water were added to make 30 L in total. The mixture was cooked for 10 minutes and then chilled to 60°C. 350 ml propionic acid and 64 g Nipagin in 350 ml ethanol were added.

NP-40 lysis buffer: 150 mM NaCl, 1% NP-40, and 50 mM Tris-HCl (pH = 7.5-8) solution was prepared and stored at 4C. Right before use, phosphatase (20mM NaF, 50mM sodium orthovanadate) and protease inhibitors were added (1 tablet protease inhibitors per 10 ml, Roche, EDTA-free).

Urea Binding Buffer for denaturing conditions: 4.8g urea was dissolved in water, 0.4 ml 500mM Tris-HCl, 3.3 ml 1500mM NaCl, and 0.25ml 200mM imidazole to make the total volume of 10 ml. The solution's pH was adjusted to 8 and 0.7 µl of β-mercaptoethanol was added.

Urea Elution Buffer for denaturing conditions: 4.8g urea was dissolved in water, 0.4 ml 500mM Tris-HCl, 3.3 ml 1500mM NaCl, and 1 ml 5M imidazole to make the total volume of 10 ml. The solution's pH was adjusted to 8 and 0.7 µl of β-mercaptoethanol was added.

TNT lysis buffer: 300 mM NaCl, 5 mM EDTA, 5 mM sodium fluoride, 2 mM sodium orthovanadate, 50 mM Tris-HCl (pH=7.5-8), and 1% Triton X-100 solution was prepared. One tablet of EDTA-free protease inhibitors per 10 ml (Roche) was added. The buffer was stored at -20 C.

Fly Squishing Buffer: 10 mM Tris (pH=8), 1 mM EDTA, and 25 mM NaCl solution was prepared and stored at room temperature. 200 µg/ml of fresh Proteinase K was added right before use. Two to five flies were squished in 50 µl of squishing buffer (on ice), placed at 37 C for 30 minutes for digestion, and at 95 C for 5 minutes to inactivate Proteinase K. 2 µl was used for PCR.

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